NF-AT POLYPEPTIDES AND POLYNUCLEOTIDES AND SCREENING METHODS FOR IMMUNOSUPPRESSIVE AGENTS

Cross-Reference to Related Applications

This application is continuation-in-part of Application No. 08/507,032, entitled "*Screening Methods for Immunosuppressive Agents*", filed on 31 July 1995, which is a file-wrapper-continuation of Application No. 08/228,944, filed on April 18, 1994, which is a file-wrapper-continuation of Application No. 07/749,385, filed on August 22, 1991; and a continuation-in-part of Application No. 08/260,174, entitled "*NF-AT Polypeptides and Polynucleotides*", filed June 13, 1994, which is a continuation-in-part of Application No. 08/124,981, entitled "*NF-AT Polypeptides and Polynucleotides*", filed September 20, 1993 (U.S. Patent No. 5,837,840). These applications are hereby incorporated by reference herein.

Statement of Rights

This invention was made in the course of work supported by the U.S. Government. The U.S. Government has therefore certain rights in this invention.

Field of the Invention

The invention provides novel polypeptides which are associated with the transcription complex NF-AT, polynucleotides encoding such polypeptides, antibodies which are reactive with such polypeptides, polynucleotide hybridization probes and PCR amplification probes for detecting polynucleotides which encode such polypeptides, transgenes which encode such polypeptides, homologous targeting constructs that encode such polypeptides and/or homologously integrate in or near endogenous genes encoding such polypeptides, nonhuman transgenic animals which comprise functionally disrupted endogenous genes that normally encode such polypeptides, and transgenic nonhuman animals which comprise transgenes encoding such polypeptides. The invention also provides methods for detecting T cells (including activated T cells) in a cellular sample, methods for treating hyperactive or hypoactive T cell conditions, methods for screening for immunomodulatory agents, methods for diagnostic staging of lymphocyte differentiation, methods for producing NF-AT proteins for use as research or diagnostic reagents, methods for producing antibodies reactive with the novel polypeptides, and methods for producing transgenic nonhuman animals.

Background of the Invention

The immune response is coordinated by the actions of cytokines produced from activated T lymphocytes, such as lymphocytes contacted with antigens. Cytokines are responsible for the control of proliferation and cell fate decisions among precursors of B cells, granulocytes and macrophages. T lymphocytes having a broad spectrum of antigen receptors are produced in the thymus as a product of the processes of selection and differentiation. When these T cells migrate to the peripheral lymphoid organs and encounter antigen, they undergo activation, during the process of which they produce large numbers of cytokines that act upon other cells of the immune system to coordinate their behavior to bring about an effective immune response.

T lymphocyte activation involves the specific regulation of many genes from minutes after the antigen encounter until at least 10 days later. T cells may also be activated by stimuli such as the combination of a calcium ionophore (e.g., ionomycin) and an activator of protein kinase C, such as phorbol myristate acetate (PMA). Several lectins, including phytohemagglutinin (PHA) may also be used to activate T cells (Nowell, P.C. (1990) *Cancer Res.* 20:462-466). The T cell activation genes are roughly grouped based on the time after stimulation at which each gene is regulated. Early genes trigger the regulation of subsequent genes in the activation pathway.

Because of the critical role of the T lymphocyte, agents that interfere with the early activation genes, such as cyclosporin A and FK506, are effective immunosuppressants. These early activation genes are regulated by transcription factors, such as NF-AT, that in turn are regulated through interactions with the antigen receptor. These transcription factors act through enhancer and promoter elements on the early activation genes to modulate their rate of transcription.

A typical early gene enhancer element is located in the first 325 base pairs upstream of the start site of the interleukin-2 gene. This region has been used extensively to dissect the requirements for T lymphocyte activation. This region binds an array of transcription factors including NF-AT, NFkB, Ap-1, Oct-1, and a newly identified protein that associates with Oct-1 called OAP-40. These different transcription factors act together to integrate the complex requirements for T lymphocyte activation.

The interleukin-2 gene is essential for both the proliferation and immunologic activation of T cells. The signaling pathways which connect the IL-2 gene and a representative and important early gene with the antigen receptor on the T cell surface and the signal transmission pathways between them are illustrated in Fig. 1. The binding site for the NF-AT protein appears to restrict expression of the interleukin-2 gene and other early activation genes to the context of an activated T lymphocyte. This information is based upon past work by Durand et al., *Mol. and Cell. Biol.* (1988), Shaw et al., *Science*, 241: 202 (1988), and Verwiej et al., (1990) *J. Biol. Chem*, 265: 15788-15795 (1990).

NF-AT appears to be the most important element among the group mentioned above in that it is able to direct transcription of any genes to activated T cells in the context of an intact transgenic animal (Verweij et al. *J. Biol. Chem.* 265:15788-15795, 1990). NF-AT is also the only element that requires physiologic activation through the antigen receptor for the activation of transcription by NF-AT. For example, the element is activated only after proper presentation of antigen of exactly the correct sequence by MHC-matched antigen presenting cells. This effect can be mimicked by pharmacologic agents, including the combination of ionomycin and PMA, which can also activate T cells through critical early genes.

Other elements within the IL-2 enhancer, for example, the NF-kB site or the AP-1 site, activate transcription in response to less specific stimuli, such as tumor necrosis factor alpha or simply PMA by itself. These compounds do not activate the IL-2 gene and other early activation genes and do not lead to T cell activation. Such observations have led to the conclusion that NF-AT restricts the expression of certain early genes, such as the interleukin-2 gene to their proper biologic context. Preliminary data have also indicated that a selective genetic deficiency of NF-AT produces severe combined immunodeficiency (SCID) (Chatilla, T. et al. *New Engl. J. Med.* 320:696-702, 1989).

As noted above, cyclosporin A (CsA) and FK506 are capable of acting as immunosuppressants. These agents inhibit T and B cell activation, mast cell degranulation and other processes essential to an effective immune response (Borel et al. (1976) *Agents Actions* 6: 468; Sung et al. (1988) *J. Exp. Med.* 168: 1539; Gao et al. *Nature* 336: 176). In T lymphocytes, these drugs disrupt an unknown step in the transmission of signals from the T cell antigen receptor to cytokine genes that coordinate the immune response (Crabtree et al. (1989) *Science* 243: 355; Schreiber et al. (1989) *Science* 251:283; Hohman & Hutlsch (1990) *New Biol.* 2: 663).

Putative intracellular receptors for FK506 and CsA have been described and found to be cis-trans prolyl isomerases (Fischer & Bang (1985) *Biochim. Biophys. Acta* 828: 39; Fischer et al. *Nature* 337: 476; Handschumacher et al. (1984) *Science* 226: 544; Lang & Schmid (1988) *Nature* 331: 453; Standaert et al. (1990) *Nature* 346: 671). Binding of the drugs inhibits isomerase activity; however, studies with other prolyl isomerase inhibitors (Bierer et al. (1990) *Science* 250: 556) and analysis of cyclosporin-resistant mutants in yeast suggest that the prevention of T lymphocyte activation results from formation of an inhibitory complex involving the drug and the isomerase (Bierer et al. (1990) *Proc. Natl. Acad. Sci. U.S.A.* 87: 9231; Tropschug et al. (1989) *Nature* 342: 953), and not from inhibition of the isomerase activity per se.

The transcription factor NF-AT appears to be a specific target of cyclosporin A and FK506, since transcription directed by this protein is completely blocked in T cells treated with these drugs, with little or no effect on other transcription factors, such as AP-1 and NF-$_K$B (Shaw et al.(1988)

op.cit; Emmel et al. (1989) *Science* 246: 1617; Mattila et al. (1990) *EMBO J.* 9: 4425). However, the drugs' actual mechanism of action remains unclear. Unfortunately, while both are potent immunosuppressive agents, neither cyclosporin nor FK506 are ideal drugs.

For example, cyclosporin adverse reactions include renal dysfunction, tremors, nausea and hypertension. Indeed, for many years researchers have attempted to develop superior replacements, with FK5O6 being the most recent candidate. Without understanding how cyclosporin (or FK506) functions at the intracellular level, developing improved immunosuppressants represents an extremely difficult research effort with a very limited likelihood of success.

Thus, there exists a significant need to understand the functional basis of cyclosporin and FK506 effectiveness. With such knowledge, improved assays for screening drug candidates would become feasible, which could in turn dramatically enhance the search process. The present invention fulfills these and other needs.

Summary of the Invention

The present invention provides novel methods and compositions useful, e.g., in screening for immunosuppressive agents. The invention is based in part on the discovery of the overall mechanism by which NF-AT is formed intracellularly from nuclear and cytoplasmic subunits and on the isolation of nucleic acids encoding NF-AT proteins.

A basis of the present invention is the discovery that NF-AT (i.e., a complex comprising $NF\text{-}AT_c$ and $NF\text{-}AT_n$) is formed when a signal from the antigen receptor induces a preexisting cytoplasmic NF-AT submit ($NF\text{-}AT_c$) to translocate to the nucleus and combine with a nuclear NF-AT subunit ($NF\text{-}AT_n$). Cyclosporin A and FK506 block translocation of the cytoplasmic component without affecting the nuclear subunit. A plausible synthesis of these studies and previous work posits that the prolyl isomerases, FK506-binding protein (FK-BP) and cyclophilin, also function to import proteins to the nucleus.

The invention is also based on the purification of two related proteins, NF-ATc and NF-ATp, encoded by separate genes that represent the preexisting or cytosolic components of NF-AT. The carboxy-terminal half of $NF\text{-}AT_c$ shows limited similarity to the DNA binding and dimerization regions of the Dorsal/Rel family of transcription factors (Fig. 15, for review, Nolan and Baltimore (1992) *Current Biology, Ltd.* 2: 211-220) however, $NF\text{-}AT_c$ appears to be the most distantly related member of the group. Expression of a full length cDNA for one of these proteins, $NF\text{-}AT_c$, activates the IL-2 promoter in non-T lymphocytes, while a dominant negative of $NF\text{-}AT_c$ specifically blocks activation of the IL-2 promoter in T lymphocytes, indicating that $NF\text{-}AT_c$ is required for IL-2 gene expression and is responsible for the restricted expression of IL-2. $NF\text{-}AT_c$

RNA expression is largely restricted to lymphoid tissues and is induced upon cell activation. The second protein, NF-AT_p, is highly homologous to NF-AT_c over a limited domain, but exhibits wider tissue distribution and is highly expressed in tissues characterized by Ca++-dependent regulation. Together these proteins are members of a new family of DNA binding proteins, which are distantly related to the Dorsal/Rel family (Nolan and Baltimore (1992) *Current Biology, Ltd.* 2: 211-220). Agents that increase intracellular Ca++ or that activate protein kinase C independently produce alterations in the mobility of NF-AT_c, indicating that distinct signaling pathways converge on NF-AT_c to regulate its function.

In accordance with one aspect of the invention, novel compositions include NF-ATc polypeptides, nuclear components of NF-AT complexes, e.g, an NF-AT_n polypeptide, mixtures of the polypeptides, and cellular extracts containing the polypeptides. The NF-AT_n and NF-AT_c subunits are capable of forming a native NF-AT complex which binds in a sequence-specific manner to a transcriptional regulatory DNA sequence of an immune response gene. The NF-AT_n subunit is characterized by:

i. a molecular weight of about 45kd;

ii. inducible expression in T cells (such as Jurkat cells);

iii. inducible expression in HeLa cells by exposing the cells to an agent (such as PMA) capable of activating protein kinase C;

iv. a lack of effect by cyclosporin and FK506 on NF-AT_n synthesis in T cells; and

v. specifically binding to an NF-AT_c.

The NF-AT_c subunit is characterized by:

i. a molecular weight of about 90kd;

ii. constitutively expressed in T cells;

iii. ability to be transported into a nucleus after a Ca++ flux in the cell;

iv. nuclear transport inhibited by cyclosporin and FK506; and

v. specifically binding to an NF-AT_n.

In another aspect of the present invention, isolated or purified nucleic acid sequences (or their complementary sequences) are provided which are capable of binding to an NFAT complex, wherein the sequences are substantially homologous to an enhancer, such as IL-2 and IL-4 enhancers, particularly the sequence AAGAGGAAAAA (SEQ ID NO: 53).

In another aspect, the invention embraces methods of screening for an immune regulating agent comprising combining the agent with a component selected from the group consisting of an NF-AT_n polypeptide, an NF-AT_c polypeptide, and mixtures thereof; and determining whether the agent binds to the selected component.

In another embodiment, candidate immunomodulatory agents are identified by their ability to block the binding of a NF-AT_c polypeptide to other components of NF-AT (e.g., AP-1) and/or to block the binding of NF-AT to DNA having an NF-AT recognition site. The DNA preferably includes one or more NF-AT binding sites at which a NF-AT protein complex specifically binds. One means for detecting binding of a NF-AT protein comprising NF-AT_c to DNA is to immobilize the DNA, such as by covalent or noncovalent chemical linkage to a solid support, and to contact the immobilized DNA with a NF-AT protein complex comprising a NF-AT_c polypeptide that has been labeled with a detectable marker (e.g., by incorporation of radiolabeled amino acid). Such contacting is typically performed in aqueous conditions which permit binding of a NF-AT protein to a target DNA containing a NF-AT binding sequence. Binding of the labeled NF-AT to the immobilized DNA is measured by determining the extent to which the labeled NF-AT_c polypeptide is immobilized as a result of a specific binding interaction. Such specific binding may be reversible, or may be optionally irreversible if a cross-linking agent is added in appropriate experimental conditions.

In yet another embodiment, methods of screening for an immune regulating agent will comprise the steps of:

i. preparing a collection of eukaroytic cells containing NF-AT_c in the cytoplasm of the cell;

ii. treating the cells with an agent;

iii. assaying for nuclear translocation of the NF-AT_c wherein blocking of nuclear transport correlates with the immunosuppressive activity of the agent. The step of assaying for nuclear translocation preferably comprises determining the nuclear presence of the NF-AT_c which is labeled with a detectable marker. Alternatively, the step of assaying for nuclear translocation comprises determining nuclear association between the NF-AT_c and an NF-AT_n, preferably using nuclei treated previously with the agent.

The assaying step can also comprise determining binding of NF-AT to a DNA sequence in the cell, such as by determining mRNA transcription levels in the cell, wherein the mRNA encodes an immune response gene.

In a different embodiment, the method of screening for immune regulating agents can comprise:

i. constructing a chimeric gene comprising an NF-AT regulated enhancer region linked to a reporter gene (e.g., chloramphenicol acetyltransterase (CAT) gene);

ii. inserting the chimeric gene into T cells;

iii. treating the T cells with T cell activating compounds in the presence or absence of the agent; and

iv. determining the effect of the agent on expression of the reporter gene. Inhibition of expression of the reporter gene indicates that the agent is a candidate immunosuppressant agent.

In one aspect, candidate immunomodulatory agents are identified as being agents capable of inhibiting (or enhancing) intermolecular binding between NF-AT_c and other polypeptides which comprises a NF-AT complex (e.g., AP-1, JunB, etc.). The invention provides methods and compositions for screening libraires of agents for the capacity to interfere with binding of NF-AT_c to other NF-AT polypeptide species under aqueous binding conditions. Typically, at least either NF-AT_c and/or another NF-AT polypeptide species is labeled with a detectable label and intermolecular binding between NF-AT_c and other NF-AT polypeptide species is detected by the amount of labeled species captured in NF-AT complexes and the like.

For example, methods of assaying for a candidate-immunosuppressant agent comprise mixing the agent with NF-AT_n and NF-AT_C under conditions which permit specific multimerization to form NF-AT, comprising dimerization of NF-AT_n and NF-AT_c, and determining whether said dimerization (and/or multimerization with other subunits) occurs. Typically, NF-AT_n or NF-AT_c, is immobilized and at least one subunit is labeled with a detectable marker, most usually the non-immobilized NF-AT subunit is labeled.

The present invention further provides several novel methods and compositions for modulating the immune response and for screening for modulators of the immune response which utilize polynucleotide sequences encoding NF-AT_c recombinant proteins and complementary polynucleotides which are substantially identical to NF-AT_c polynucleotide sequences.

Thus, in another aspect of the invention, NF-AT_c polypeptides comprising polypeptide sequences which are substantially identical to a sequence shown in Fig. 12 or a cognate NF-AT_c amino acid sequence are provided.

The invention also provides for nucleic acid sequences encoding NF-AT_c. The characteristics of the cloned sequences are given, including the nucleotide and predicted amino acid sequence in Fig. 12. Polynucleotides comprising these sequences can serve as templates for the recombinant expression of quantities of NF-AT_c polypeptides, such as human NF-AT_c and murine NF-AT_c. Polynucleotides comprising these sequences can also serve as probes for nucleic acid hybridization to detect the transcription and mRNA abundance of NF-AT_c mRNA in individual lymphocytes (or other cell types) by *in situ* hybridization, and in specific lymphocyte populations by Northern blot analysis and/or by *in situ* hybridization (Alwine et al. (1977) *Proc. Natl. Acad. Sci. U.S.A.* 74: 5350) and/or PCR amplification and/or LCR detection. Such recombinant

polypeptides and nucleic acid hybridization probes have utility for *in vitro* screening methods for immunomodulatory agents and for diagnosis and treatment of pathological conditions and genetic diseases, such as transplant rejection reactions, T cell-mediated immune responses, lymphocytic leukemias (e.g., T cell leukemia or lymphoma) wherein NF-AT activity contributes to disease processes, autoimmune disease, arthritis, and the like.

The invention also provides antisense polynucleotides complementary to NF-AT_c sequences which are employed to inhibit transcription and/or translation of the cognate mRNA species and thereby effect a reduction in the amount of the respective NF-AT_c protein in a cell (e.g., a T lymphocyte of a patient). Such antisense polynucleotides can function as immunomodulatory drugs by inhibiting the formation of NF-AT protein required for T cell activation.

In a variation of the invention, polynucleotides of the invention are employed for diagnosis of pathological conditions or genetic disease that involve T cell neoplasms or T cell hyperfunction of hypofunction, and more specifically conditions and diseases that involve alterations in the structure or abundance of NF-AT_c polypeptide, NF-AT_c polynucleotide sequence, or structure of the NF-AT_c gene or flanking region(s).

The invention also provides antibodies which bind to NF-AT_c with an affinity of about at least 1×10^7 M^{-1} and which lack specific high affinity binding for other proteins present in activated T cells. Such antibodies can be used as diagnostic reagents to identify T cells (e.g., activatable T cells) in a cellular sample from a patient (e.g., a lymphocyte sample, a solid tissue biopsy) as being cells which contain an increased amount of NF-AT_c protein determined by standardization of the assay to be diagnostic for activated T cells. Frequently, anti-NF-AT_c antibodies are included as diagnostic reagents for immunohistopathology staining of cellular samples *in situ*. Additionally, anti-NF-AT_c antibodies may be used therapeutically by targeted delivery to T cells (e.g., by cationization or by liposome/immunoliposome delivery).

The invention also provides NF-AT_c polynucleotide probes for diagnosis of neoplasia or immune status by detection of NF-AT_c mRNA in cells explanted from a patient, or detection of a pathognomonic NF-AT_c allele (e.g., by RFLP or allele-specific PCR analysis). A pathognomonic NF-AT_c allele is an allele which is statistically correlated with the presence of a predetermined disease or propensity to develop a disease. Typically, the detection will be by in situ hybridization using a labeled (e.g., ^{32}P, ^{35}S, ^{14}C, ^{3}H, fluorescent, biotinylated, digoxigeninylated) NF-AT_c polynucleotide, although Northern blotting, dot blotting, or solution hybridization on bulk RNA or poly A^+ RNA isolated from a cell sample may be used, as may PCR amplification using NF-AT_c-specific primers. Cells which contain an increased amount of NF-AT_c mRNA as compared to standard control values for cells or cell types other than activated T cells or activatable T cells will be thereby identified as activated T cells or activatable T cells. Similarly, the detection of

pathognomonic rearrangements or amplification of the NF-AT_c locus or closely linked loci in a cell sample will identify the presence of a pathological condition or a predisposition to developing a pathological condition (e.g., cancer, genetic disease).

The present invention also provides a method for diagnosing T cell hypofunction of hyperfunction in a human patient, wherein a diagnostic assay (e.g., immunohistochemical staining of fixed lymphocytic cells by an antibody that specifically binds human NF-AT_c) is used to determine if a predetermined pathognomonic concentration of NF-AT_c protein or NF-AT_c mRNA is present in a biological sample from a human patient; if the assay indicates the presence of NF-AT_c protein or NF-AT_c mRNA at or above such predetermined pathognomonic concentration, the patient is diagnosed as having T cell hyperfunction or hypofunction condition, or transplant rejection and the like.

All publications and patent applications herein are incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Brief Description of the Drawings

Figure 1. Representation of the signal transmission pathways carrying information from the T lymphocyte antigen receptor to the early activation genes that lead to proliferation, clonal expansion and immune function or to cell death (apoptosis), clonal deletion and tolerance. Primary signals emanate from the interaction of the T cell antigen receptor (includes the TCR and CD3 complex) with antigen bound by the major histocompatibility complex (MHC). Accessory signaling molecules such as CD2, CD4, and LFA-1 augment the primary signal. A secondary signal that is required to completely activate T lymphocytes is provided by interleukins 1 and 6. These initial signals are transmitted to the nucleus by second messengers such as tyrosine phosphatases (CD-45), tyrosine kinases (lck and fyn), as well as by protein kinase C (PKC) and intracellular calcium. As depicted in the schematic, immunosuppressive drugs such as FK506 and cyclosporin (CsA) as well as immune deficiency diseases (SCID) interfere with the proper transmission of signals from the TCR to the nucleus.

Figure 2. Diagram of the human IL-2 enhancer from -326 to +47 base pairs. DNase I protected regions are noted by boxes along with the identification of the sites (A-E) and the name(s) of the proteins which complex with these sites. Mutations introduced in the boxed regions drastically reduce IL-2 transcription following T lymphocyte activation and are indicated as percent wild type (full) expression remaining. The arrow identifies the transcriptional start site.

Figure 3. The diagram shows the NF-AT binding site from the distal element in the interleukin-2 gene. Contact guanine residues are indicated by lower case letters and the

construction of binding sites used to measure NF-AT dependent transcriptional activity are shown as an array of three NF-AT binding sites.

Figure 4. NF-AT is T cell enriched and is formed following activation of T lymphocytes. Representation of NF-AT in different cell lines. Nuclear extracts from: J, Jurkat cells; K, KB cells (a derivative of HeLa cells); F, Faza cells (a rat liver cell line); H, Hep G2 cells (a human hepatocyte line); T, TEPC murine B-cell line; E, EL-4 murine T cell line; C, C2Cl2 murine myoblasts. Lanes labelled "+" are the complexes formed with nuclear extracts from cells treated with PHA (2 μg/ml) and PMA (50 ng/ml) for two hours.

Figure 5. The nuclear component of the NF-AT complex requires protein synthesis while the cytoplasmic component of NF-AT is pre-existing in T cells. Lanes 1 and 2, gel mobility shift assay using nuclear extracts (10 μg) from stimulated/FK5060 - treated (s+F) cells in the presence (+) and absence (-) of 100 μM anisomycin. Lanes 3-6, complementation of nuclear extracts from stimulated/FK506-treated cells (with or without anisomycin pretreatment) with cytoplasmic extracts prepared from nonstimulated cells treated with or without anisomycin. Arrows indicate the mobility of the reconstituted NF-AT DNA binding complex.

Figure 6. NF-AT binding activity can be quantitatively reconstituted from nuclear and cytoplasmic fractions of stimulated/FK506- and stimulated/CsA-treated Jurkat cells. (a) In lanes 1-6, nuclear extracts (10 μg) and cytoplasmic extracts (10 μg) from nonstimulated (ns), stimulated (s) with PMA/ionomycin, and stimulated/FK506-treated (s+F) cells were tested for NF-AT binding activity using electrophoretic gel mobility shift assays. In lanes 7-10, NF-AT was reconstituted by mixing nuclear and cytoplasmic extracts. Stimulated/FK506-treated (s+F) nuclear extracts (5 μg) were complemented with cytoplasmic extracts (5 μg) from: lane 7, nonstimulated (ns); lane 8, stimulated (s); lane 9, stimulated/FK506-treated (s+F); and lane 10, stimulated/rapamycin-treated (rap) cells. In all cases arrows indicate the NF-AT protein DNA complex. (b) In lanes 1-3, mixing of nuclear extracts from nonstimulated cells (5 μg) with any cytoplasmic extracts (5 μg) fails to reconstitute NF-AT binding. In lanes, 4-9, reconstituted NF-AT binding activity demonstrates DNA binding specificity: nuclear extracts (5 μg) from stimulated/FK506-treated cells (s+F) were mixed with cytoplasmic extracts (5 μg) and competition was carried out with 10 ng of unlabeled NF-AT or mutant NF-AT oligonucleotide. (c) The effect of FK506 was tested on a murine T cell hybridoma, JK12/90.1 (Karttunen et al. (1991) *PNAS* 88:3972). In lanes 1-3, nuclear extracts (10 μg) from nonstimulated/FK506-treated (s+F) Jurkat cells were tested for NF-AT binding activity. Lane 6 shows NF-AT binding in nuclear extracts from stimulated/FK506treated (s+F) JK12/90.1 cells. In lanes 4-5 and 7-8, NF-AT is reconstituted by mixing nuclear extracts (5 μg) from stimulated/FK506-treated Jurkat or JK12/90.1 cells with cytoplasmic extracts (5 μg) from nonstimulated Jurkat or JK12/90.1 cells. (d) In lanes 1-3, nuclear extracts (5 μg) from

nonstimulated (ns), stimulated (s), and stimulated/cyclosporin A-treated (s+C) cells. Stimulated/cyclosporin A-treated (s+C) nuclear extracts (5 μg) were complemented with cytoplasmic fractions (5 μg) from: lane 4, nonstimulated (ns); lane 5, stimulated (s); and lane 6, stimulated/cyclosporin A-treated (s+C) cells.

Figure 7. The nuclear component of NF-AT is present in HeLa cells and can be complemented by Jurkat cytoplasm, but not by HeLa cell cytoplasm, to reconstitute NF-AT binding activity. (a) Lanes 1-6, gel mobility shift assay using HeLa nuclear (10 μg) and cytoplasmic (10 μg) extracts from nonstimulated (ns), stimulated (s), and stimulated/FK506-treated (s+F) cells do not form a NF-AT protein-DNA complex. In lanes 7-9, homologous mixing of nuclear extracts (5 μg) from stimulated/FK506-treated (s+F) HeLa cells with HeLa cytoplasmic extracts (5 μg) does not reconstitute NF-AT. (b) NF-AT binding activity is reconstituted with HeLa nuclear and Jurkat cytoplasmic extracts. Nuclear extracts (5 μg) from stimulated/FK506treated HeLa cells complemented by cytoplasmic extracts (5μ) from: lane 1, nonstimulated (ns); and lane 3, stimulated/FK506-treated (s+F) Jurkat cells. In lanes 4-9, reconstituted NF-AT binding complex demonstrates DNA binding specificity when competed by 10 μg of unlabelled NF-AT or mutant NF-AT oligonucleotides. (c) Lanes 1-3, heterologous mixing of Jurkat nuclear extracts (5 μg) from stimulated/FK506treated (s+F) with HeLa -cytoplasmic extracts (5 μg) does not reconstitute NF-AT binding activity.

Figure 8. The nuclear component of NF-AT is induced by PMA while calcium mediated signals allow translocation of the preexisting cytoplasmic subunit of NF-AT. (a) Lanes 1 and 2, gel mobility shift assay using nuclear extracts (10 μg) from PMAstimulated (p) and ionomycin-stimulated (i) cells. In lanes 3-8, complementation of nuclear extracts (5 μg) from PMAstimulated and ionomycin-stimulated cells with cytoplasmic extracts (5 μg) from nonstimulated (ns), stimulated (s), and stimulated/FK506-treated (s+F) cells. Stimulated/FK506-treated (s+F) nuclear extracts (5 μg) were complemented with cytoplasmic extracts (5 μg) from: lane 9, nonstimulated (ns); lane 10, PMA-stimulated (p); lane 11, ionomycin-stimulated (i); and lane 12, stimulated (s) cells. Arrows indicate the NF-AT protein DNA binding complex.

Figure 9. In vitro transcription directed by the IL-2 enhancer or three tandemly linked NF-AT binding sites in nuclear extracts stimulated under different conditions. (a) IL-2 directed transcription: lane 1, nonstimulated; lane 2, PMA/ ionomycin/FK506-treated cells; and PMA/ionomycin stiraulated cells, lane 3. NF-AT directed transcription: lane 4,cdescriptionde nonstimulated; lane 5, PMA/ionomycin/FK506treated cells; lane 6, PMA/ionomycin-stimulated cells. Expression from the IL-2 enhancer and NF-AT G-less template generates a 401 and 383 nucleotide (nt) transcript, respectively. The adenovirus major late promoter (AdMLP) internal control generates a 280 nt transcript. Fold induction is calculated following normalization to

AdMLP transcription. (b) Ribonuclease protection assay of NF-AT driven lac-Z mRNA. Lane 1, nonstimulated; lane 2, PMA/ionomycin-stimulated; lane 3, PMA/ionomycin/FK506-treated cells.

Figure 10. NF-AT dependent T-antigen transcription levels in tissues of transgenic mice. Total RNA was prepared from tissues of a 6-week-old mouse of line Tag8 (Verweij et al. *JBC* 265:15788-15795 (1990)). Spleen, thymus and bone marrow cells were cultured for 24 hours in the presence of ionomycin (0.6 μM1 and PMA (10 μg/ml). Ten micrograms of each RNA sample was used in an RNase protection assay. As a probe we used the 176 nucleotide P-32 labeled antisense NF-AT-Tag RNA probe. Correctly initiated mRNA would yield a 47-nucleotide protected fragment. The position of the fragment (TI) is indicated by an arrow.

Figure 11. Dephosphorylation of NF-AT inhibits its DNA binding. Lanes 1-5, gel mobility shift assay. Nuclear extracts (10 μg) from PMA/ionomycin stimulated Jurkat cells were incubated with several protein phosphatase inhibitors in the presence or absence of calf intestinal phosphatase. Characteristic NF-AT mobility shift in the presence of: lane 1, 200 μm sodium vanadate (Na_2VO_4); lane 2, 200 mM sodium molybdate (Na_2MO_4) ; lane 3, 10 mM sodium fluoride (NaF); lane 4, one unit of calf intestinal phosphatase (CIP); lane 5, one unit of calf intestinal phosphatase plus 200 μM of sodium vanadate, 200 μM sodium molybdate and 10 mm sodium fluoride. For methods see Figure 6. The arrow indicates the NF-AT protein DNA complex.

Figure 12 Panels A-G (SEQ ID NOs: 45-46) shows the nucleotide sequence of the human NF-AT_c cDNA and the deduced amino acid sequence. N indicates that a sequence ambiguity is present.

Figure 13 shows the expression of NF-AT_c protein in T cells (Jurkat) and non-T cells (Cos).

Figure 14 Panels A and B show that the NF-AT_c cDNA clone encodes a protein that activates transcription from an NF-AT site and is capable of activating the IL-2 promoter in non-T cells.

Fig. 15 (SEQ ID Nos: 47-52) shows the homology between NF-AT_c, NF-AT_p, and Rel family members. The protein sequences of murine NF-AT_p and the Rel proteins Dorsal (the Drosophila axis-determining protein), human c-Rel, NF-*K*B p50, and NF-*K*B p65 are aligned to the sequence of NF-AT_c. Numbering is with respect to NF-AT_c. Identity to NF-AT_c, open boxes; similarity in known residue function or structure, shaded areas. Stars indicate regions in which NF-AT_c has: 1) a charge reversal relative to the majority of other Rel proteins, or has 2) replaced a potential salt bridge residue with a histidine or other chelating residue. Lower portion shows a schematic of NF-AT_c and NF-AT_p.

Figure 16 (panels A-C). Panel A: Ribonuclease protection for human NF-AT_c with RNA from Jurkat cells (lanes 1-6) or Hela cells (lane 7). The expected specific ribonuclease-resistant fragment is 304 nucleotides (arrow). Hela cells were non-stimulated. Jurkat cells were either non-

stimulated or stimulated with 20ng/ml PMA and 2uM ionomycin for 3 hours, plus or minus 100ng/ml CsA added at the indicated times after stimulation. Panel B: RNA from the following human cells: KJ (preB cell ALL), JD-1 (B cell lineage ALL), K562 (erythroleukemia cell line), CML (bone marrow cells from a patient with a myeloid leukemia), human muscle tissue, Hep G2 (liver cell line), HPB ALL (T cell line, nonstimulated or stimulated with 2ug/mi PHA and 50ng/ml PMA for 30 minutes), and Hela cells analyzed by ribonuclease protection. A longer exposure of this gel indicates that the K562 cell line contains a small amount of NF-AT_c transcript. Panel C: NF-AT_c (upper panel) and NF-AT_p (lower panel) mRNA expression in mouse tissues and a skin tumor derived from NF-AT-Tag transgenic mice (Verweij et al. (1990) *J. Biol. Chem* 265: 15788-15795). Cells were either non-stimulated or stimulated with 20ng/ml PMA and 2uM ionomycin for 3 hours. RNA was measured by quantiative ribonuclease protection using murine cDNA probes. The predicted size of the fragment homologous to the probe is indicated by the arrows.

Figure 17 (panels A-D). Panel A: Cos cells and Jurkat cells were transfected with reporter constructs for NF-AT or HNF-1 (*β28*). Co-transfected expression vectors for NF-AT_c (+NF-AT) or HNF-1*α*(+HNF-1) were included where indicated, otherwise empty pBJ5 vector was included. Cells were stimulated as indicated: PMA, P + I (PMA plus ionomycin). Panel B: Cos cells were transfected with IL-2 luciferase and with expression vectors as in A. Stimulations were as in A. Data in A and B are expressed as fold induction of luciferase activity over nonstimulated value with empty pBJ5 vector. Bars represent mean and range of 2-3 independent transfections. Panel C: Expression of NF-AT_c in Cos cells gives rise to specific DNA binding activity. Gel mobility shifts using nuclear extracts from Cos cells transfected with pBJ5 (lanes 1 and 3), with NF-AT_c (lanes 2 and 4-7), from non-transfected Jurkat cells (lanes 8-11) or using cytosols from pBJ5- or NF-AT_c-transfected Cos cells (lanes 12-13, 15-16) combined with Hela nuclear extract (lanes 15-16). Lane 14, Hela nuclear extract alone. Labeled AP-1 (lanes 1-2) or NF-AT (lanes 3-16) probes and cold competitor oligonucleotides are indicated. Arrows indicate specific AP-1 and NF-AT complexes. Panel D: Antisera induced supershift of NF-AT. NF-AT and AP-1 gel mobility shifts using nuclear extracts from stimulated Jurkat cells or murine thymocytes. Either no antisera, preimmune, or one of two different immune antisera was included as indicated. Arrows indicate specific NF-AT or AP1 complexes or supershifted NF-AT complexes (*).

Figure 18 shows dominant-negative NF-AT_c. Jurkat Tag cells were transfected with vector plasmid (control) or with the dominant negative NF-AT_c plasmid, plus the indicated secreted alkaline phosphatase reporter plasmid. Transfected cells were transferred to fresh culture medium 24 hours after transfection and secreted alkaline phosphatase activity was measured (Clipstone and Crabtree (1992) *Nature* 357: 695-698) 16 to 24 hours later, after stimulation with 1 uM ionomycin plus 20 ng/ml PMA (NF-AT and IL-2 reporters), 20 ng/ml PMA alone (AP-1 reporter) or no

stimulation (RSV reporter). Bars indicate, secreted alkaline phosphatase activity from cells transfected with the dominant negative NF-AT_c as a percentage of the activity from cells transfected in parallel with control plasmid, and represent data obtained from (n) independent transfections. The dominant negative NF-AT_c consists of a carboxy terminal truncation of the epitope tagged NF-AT_c expression plasmid extending to the PvuII site at amino acid 463.

Figure 19 shows changes in mobility of epitope tagged NF-AT_c expressed in Jurkat cells. Cells were transfected with NF-AT_c as in Fig. 13 and stimulated as shown for 2 hrs plus or minus 100ng/ml CsA. Whole cell lysates were analyzed by western blotting as in Fig. 13.

Detailed Description of the Invention

The present invention pertains to means of modulating transcription that is dependent upon the presence of a linked cis-acting NF-AT site as well as methods of causing and preventing formation of transcriptionally active NF-AT complexes, controlling expression of the early T lymphocyte activation genes, and controlling transcription of the human immunodeficiency virus. The invention also relates to the formation of active NF-AT from nuclear and cytoplasmic subunits by a novel mechanism; control of induction of the nuclear precursor of NF-AT, as well as control of the nuclear import of the cytoplasmic precursor of NF-AT, methods by which the nuclear import of NF-AT can be modulated and methods by which the induction of the nuclear subunit of NF-AT can be prevented or enhanced. The methods of this invention are useful in determining or controlling the expression of early T lymphocyte activation genes as well as determining or controlling the expression of selected constitutive genes that can be advantageously expressed in T lymphocytes. In addition, the invention also pertains to the development of screening assays for agents that modulate the nuclear import of the cytoplasmic subunit of NF-AT or the induction of the nuclear subunit of NF-AT, such agents are thereby identified as candidate immunosuppressant agents.

A basis of the present invention is the experimental finding that multimeric-complexes are formed when a signal from the antigen receptor induces a pre-existing cytoplasmic subunit to translocate to the nucleus and combine with a newly synthesized nuclear subunit of NF-AT. Formation of a functional multimeric complex, which includes [NF-AT_c:NF-AT_n] heterodimer, then facilitates transcriptional enhancement by interacting with specific NF-AT recognition sequences near particular structural genes, such as the IL-2 gene. Since transcriptional enhancement of early genes, such as the IL-2 gene, is a critical step in the process of T lymphocyte activation, candidate immunosuppressants can be identified by screening for agents which interfere with the formation of functional NF-AT heterodimer and/or inhibit transcriptional enhancement that entails NF-AT interacting with specific NF-AT recognition sequences.

Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Generally, the nomenclature used hereafter and the laboratory procedures in cell culture, molecular genetics, and nucleic acid chemistry and hybridization described below are those well known and commonly employed in the art. Standard techniques are used for recombinant nucleic acid methods, polynucleotide synthesis, and microbial culture and transformation (e.g., electroporation, lipofection). Generally enzymatic reactions and purification steps are performed according to the manufacturer's specifications. The techniques and procedures are generally performed according to conventional methods in the art and various general references (see, generally, Sambrook et al. *Molecular Cloning: A Laboratory Manual*, 2d ed. (1989) Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., which is incorporated herein by reference) which are provided throughout this document. The procedures therein are believed to be well known in the art and are provided for the convenience of the reader. All the information contained therein is incorporated herein by reference.

Oligonucleotides can be synthesized on an Applied Bio Systems oligonucleotide synthesizer according to specifications provided by the manufacturer.

Methods for PCR amplification are described in the art *(PCR Technology: Principles and Applications for DNA Amplification* ed. HA Erlich, Freeman Press, New York, NY (1992); *PCR Protocols: A Guide to Methods and Applications*, eds. Innis, Gelfland, Snisky, and White, Academic Press, San Diego, CA (1990); Mattila et al. (1991) *Nucleic Acids Res.* 19: 4967; Eckert, K.A. and Kunkel, T.A. (1991) *PCR Methods and Applications* 1: 17; *PCR*, eds. McPherson, Quirkes, and Taylor, IRL Press, Oxford; and U.S. Patent 4,683,202, which are incorporated herein by reference).

Although any methods and materials similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described. For purposes of the present invention, the following terms are defined below.

1. Definitions

As used herein, the twenty conventional amino acids and their abbreviations follow conventional usage (*Immunology - A Synthesis*, 2nd Edition, E.S. Golub and D.R. Gren, Eds., Sinauer Associates, Sunderland, Massachusetts (1991), which is incorporated herein by reference). Stereoisomers (e.g., D-amino acids) of the twenty conventional amino acids, unnatural amino acids such as α,α-disubstituted amino acids, N-alkyl amino acids, lactic acid, and other unconventional amino acids may also be suitable components for polypeptides of the present invention. Examples of unconventional amino acids include: 4-hydroxyproline, γ-carboxyglutamate, ϵ-N,N,N-

trimethyllysine, ϵ-N-acetyllysine, O-phosphoserine, N-acetylserine, N-formylmethionine, 3-methylhistidine, 5-hydroxylysine, ω-N-methylarginine, and other similar amino acids and imino acids (e.g., 4-hydroxyproline). In the polypeptide notation used herein, the lefthand direction is the amino terminal direction and the righthand direction is the carboxy-terminal direction, in accordance with standard usage and convention. Similarly, unless specified otherwise, the lefthand end of single-stranded polynucleotide sequences is the 5' end; the lefthand direction of double-stranded polynucleotide sequences is referred to as the 5' direction. The direction of 5' to 3' addition of nascent RNA transcripts is referred to as the transcription direction; sequence regions on the DNA strand having the same sequence as the RNA and which are 5' to the 5' end of the RNA transcript are referred to as "upstream sequences"; sequence regions on the DNA strand having the same sequence as the RNA and which are 3' to the 3' end of the RNA transcript are referred to as "downstream sequences".

The term "transcriptional enhancement" is used herein to refer to functional property of producing an increase in the rate of transcription of linked sequences that contain a functional promoter.

The term "agent" is used herein to denote a chemical compound, a mixture of chemical compounds, a biological macromolecule, or an extract made from biological materials such as bacteria, plants, fungi, or animal (particularly mammalian) cells or tissues. Agents are evaluated for potential activity as immunosupressants by inclusion in screening assays described hereinbelow.

The terms "immunosuppressant" and "immunosuppressant agent" are used herein interchangeably to refer to agents that have the functional property of inhibiting an immune response in human, particularly an immune response that is mediated by activated T cells.

The terms "candidate immunosuppressant" and "candidate immunosuppressant agent" are used herein interchangeably to refer to an agent which is identified by one or more screening method(s) of the invention as a putative inhibitor of T cell activation. Some candidate immunosuppressants may have therapeutic potential.

The term "altered ability to modulate" is used herein to refer to the capacity to either enhance transcription or inhibit transcription of a gene; such enhancement or inhibition may be contingent on the occurrence of a specific event, such as T cell stimulation. For example but not for limitation, an agent that prevents expression of NF-AT_C protein will alter the ability of a T cell to modulate transcription of an IL-2 gene in response to an antigen stimulus. This alteration will be manifest as an inhibition of the transcriptional enhancement of the IL-2 gene that normally ensues following T cell stimulation. The altered ability to modulate transcriptional enhancement or inhibition may affect the inducible transcription of a gene, such as in the just-cited IL-2 example, or may effect the basal level transcription of a gene, or both.

The term "naturally-occurring" as used herein as applied to an object refers to the fact that an object can be found in nature. For example, a polypeptide or polynucleotide sequence that is present in an organism (including viruses) that can be isolated from a source in nature and which has not been intentionally modified by man in the laboratory is naturally-occurring.

The term "corresponds to" is used herein to mean that a polynucleotide sequence is homologous (i.e., is identical, not strictly evolutionarily related) to all or a portion of a reference polynucleotide sequence, or that a polypeptide sequence is identical to a reference polypeptide sequence. In contradistinction, the term "complementary to" is used herein to mean that the complementary sequence is homologous to all or a portion of a reference polynucleotide sequence. For illustration, the nucleotide sequence "TATAC" corresponds to a reference sequence "TATAC" and is complementary to a reference sequence "GTATA".

The following terms are used to describe the sequence relationships between two or more polynucleotides: "reference sequence", "comparison window", "sequence identity", "percentage of sequence identity", and "substantial identity". A "reference sequence" is a defined sequence used as a basis for a sequence comparision; a reference sequence may be a subset of a larger sequence, for example, as a segment of a full-length cDNA or gene sequence given in a sequence listing, such as a polynucleotide sequence of Fig. 1, or may comprise a complete cDNA or gene sequence. Generally, a reference sequence is at least 20 nucleotides in length, frequently at least 25 nucleotides in length, and often at least 50 nucleotides in length. Since two polynucleotides may each (1) comprise a sequence (i.e., a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) may further comprise a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window", as used herein, refers to a conceptual segment of at least 20 contiguous nucleotide positions wherein a polynucleotide sequence may be compared to a reference sequence of at least 20 contiguous nucleotides and wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) of 20 percent or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal alignment of sequences for aligning a comparison window may be conducted by the local homology algorithm of Smith and Waterman (1981) *Adv. Appl. Math.* 2: 482, by the homology alignment algorithm of Needleman and Wunsch (1970) *J. Mol. Biol.* 48: 443, by the search for similarity method of Pearson and Lipman (1988) *Proc. Natl. Acad. Sci. (U.S.A.)* 85: 2444, by computerized implementations of these algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics

Computer Group, 575 Science Dr., Madison, WI), or by inspection, and the best alignment (i.e., resulting in the highest percentage of homology over the comparison window) generated by the various methods is selected. The term "sequence identity" means that two polynucleotide sequences are identical (i.e., on a nucleotide-by-nucleotide basis) over the window of comparison. The term "percentage of sequence identity" is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g., A, T, C, G, U, or I) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparision (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. The terms "substantial identity" as used herein denotes a characteristic of a polynucleotide sequence, wherein the polynucleotide comprises a sequence that has at least 85 percent sequence identity, preferably at least 90 to 95 percent sequence identity, more usually at least 99 percent sequence identity as compared to a reference sequence over a comparison window of at least 20 nucleotide positions, frequently over a window of at least 25-50 nucleotides, wherein the percentage of sequence identity is calculated by comparing the reference sequence to the polynucleotide sequence which may include deletions or additions which total 20 percent or less of the reference sequence over the window of comparison. The reference sequence may be a subset of a larger sequence, for example, as a segment of the full-length human NF-AT_c polynucleotide sequence shown in Fig. 12 or the full-length murine or bovine NF-AT_c cDNA sequence.

As applied to polypeptides, the term "substantial identity" means that two peptide sequences, when optimally aligned, such as by the programs GAP or BESTFIT using default gap weights, share at least 80 percent sequence identity, preferably at least 90 percent sequence identity, more preferably at least 95 percent sequence identity or more (e.g., 99 percent sequence identity). Preferably, residue positions which are not identical differ by conservative amino acid substitutions. Conservative amino acid substitutions refer to the interchangeability of residues having similar side chains. For example, a group of amino acids having aliphatic side chains is glycine, alanine, valine, leucine, and isoleucine; a group of amino acids having aliphatic-hydroxyl side chains is serine and threonine; a group of amino acids having amide-containing side chains is asparagine and glutamine; a group of amino acids having aromatic side chains is phenylalanine, tyrosine, and tryptophan; a group of amino acids having basic side chains is lysine, arginine, and histidine; and a group of amino acids having sulfur-containing side chains is cysteine and methionine. Preferred conservative amino acids substitution groups are: valine-leucine-isoleucine, phenylalanine-tyrosine, lysine-arginine, alanine-valine, and asparagine-glutamine.

The term "NF-AT_c native protein" and "full-length NF-AT_c protein" as used herein refers to a a naturally-occurring NF-AT_c polypeptide corresponding to the deduced amino acid sequence shown in Fig. 12 or corresponding to the deduced amino acid sequence of a cognate full-length cDNA. Also for example, a native NF-AT_c protein present in naturally-occurring lymphocytes which express the NF-AT_c gene are considered full-length NF-AT_c proteins.

The term "NF-AT_c fragment" as used herein refers to a polypeptide that has an amino-terminal and/or carboxy-terminal deletion, but where the remaining amino acid sequence is identical to the corresponding positions in the NF-AT_c sequence deduced from a full-length cDNA sequence (e.g., the cDNA sequence shown in Fig. 1). NF-AT_c fragments typically are at least 14 amino acids long, preferably at least 20 amino acids long, usually at least 50 amino acids long or longer.

The term "NF-AT_c analog" as used herein refers to polypeptides which are comprised of a segment of at least 25 amino acids that has substantial identity to a portion of the deduced amino acid sequence shown in Fig. 12 , and which has at least one of the following properties: (1) binding to other NF-AT proteins (e.g., AP-1) under suitable binding conditions, or (2) ability to localize to the nucleus upon T cell activation. Typically, NF-AT_c analog polypeptides comprise a conservative amino acid substitution (or addition or deletion) with respect to the naturally-occurring sequence. NF-AT_c analogs typically are at least 20 amino acids long, preferably at least 50 amino acids long or longer, most usually being as long as full-length naturally-occurring NF-AT_c (e.g., as shown in Fig. 12). Some NF-AT_c analogs may lack biological activity but may still be employed for various uses, such as for raising antibodies to NF-AT_c epitopes, as an immunological reagent to detect and/or purify α-NF-AT_c antibodies by affinity chromatography, or as a competitive or noncompetitive agonist, antagonist, or partial agonist of native NF-AT_c protein function.

The term "NF-AT_c polypeptide" is used herein as a generic term to refer to native protein, fragments, or analogs of NF-AT_c. Hence, native NF-AT_c, fragments of NF-AT_c, and analogs of NF-AT_c are species of the NF-AT_c polypeptide genus. Preferred NF-AT_c polypeptides include: the human full-length NF-AT_c protein comprising the polypeptide sequence shown in Fig. 12, or polypeptides consisting essentially of a sequence shown in Table II.

The term "cognate" as used herein refers to a gene sequence that is evolutionarily and functionally related between species. For example but not limitation, in the human genome, the human CD4 gene is the cognate gene to the mouse CD4 gene, since the sequences and structures of these two genes indicate that they are highly homologous and both genes encode a protein which functions in signaling T cell activation through MHC class II-restricted antigen recognition. Thus, the cognate murine gene to the human NF-AT_c gene is the murine gene which encodes an expressed protein which has the greatest degree of sequence identity to the human NF-AT_c protein

and which exhibits an expression pattern similar to that of the human NF-AT_c (e.g., expressed in T lineage cells). Preferred cognate NF-AT_c genes are: rat NF-AT_c, rabbit NF-AT_c, canine NF-AT_c, nonhuman primate NF-AT_c, porcine NF-AT_c, bovine NF-AT_c, and hamster NF-AT_c.

The term "NF-AT_c-dependent gene" is used herein to refer to genes which: (1) have a NF-AT binding site (a site which can be specifically footprinted by NF-AT under suitable binding conditions) within about 10 kilobases of the first coding sequence of said gene, and (2) manifest an altered rate of transcription, either increased or decreased, from a major or minor transcriptional start site for said gene, wherein such alteration in transcriptional rate correlates with the presence of NF-AT_c polypeptide in NF-AT complexes, such as in an activated T cell.

The term "candidate immunomodulatory agent" is used herein to refer to an agent which is identified by one or more screening method(s) of the invention as a putative immunomodulatory agent. Some candidate immunomodulatory agents may have therapeutic potential as drugs for human use.

As used herein, the terms "label" or "labeled" refers to incorporation of a detectable marker, e.g., by incorporation of a radiolabeled amino acid or attachment to a polypeptide of biotinyl moieties that can be detected by marked avidin (e.g., streptavidin containing a fluorescent marker or enzymatic activity that can be detected by optical or colorimetric methods). Various methods of labeling polypeptides and glycoproteins are known in the art and may be used. Examples of labels for polypeptides include, but are not limited to, the following: radioisotopes (e.g., ^{3}H, ^{14}C, ^{35}S, ^{125}I, ^{131}I), fluorescent labels (e.g., FITC, rhodamine, lanthanide phosphors), enzymatic labels (e.g., horseradish peroxidase, β-galactosidase, luciferase, alkaline phosphatase), biotinyl groups, predetermined polypeptide epitopes recognized by a secondary reporter (e.g., leucine zipper pair sequences, binding sites for secondary antibodies, metal binding domains, epitope tags). In some embodiments, labels are attached by spacer arms of various lengths to reduce potential steric hindrance.

As used herein, "substantially pure" means an object species is the predominant species present (i.e., on a molar basis it is more abundant than any other individual species in the composition), and preferably a substantially purified fraction is a composition wherein the object species comprises at least about 50 percent (on a molar basis) of all macromolecular species present. Generally, a substantially pure composition will comprise more than about 80 to 90 percent of all macromolecular species present in the composition. Most preferably, the object species is purified to essential homogeneity (contaminant species cannot be detected in the composition by conventional detection methods) wherein the composition consists essentially of a single macromolecular species.

As used herein the terms "pathognomonic concentration", "pathognomonic amount", and "pathognomonic staining pattern" refer to a concentration, amount, or localization pattern, respectively, of a NF-AT_c protein or mRNA in a sample, that indicates the presence of a hypofunctional or hyperfunctional T cell condition or a predisposition to developing a disease, such as graft rejection. A pathognomonic amount is an amount of a NF-AT_c protein or NF-AT_c mRNA in a cell or cellular sample that falls outside the range of normal clinical values that is established by prospective and/or retrospective statistical clinical studies. Generally, an individual having a neoplastic disease (e.g., lymphocytic leukemia) or T cell-mediated immune response will exhibit an amount of NF-AT_c protein or mRNA in a cell or tissue sample that is higher than the range of concentrations that characterize normal, undiseased individuals; typically the pathognomonic concentration is at least about one standard deviation above the mean normal value, more usually it is at least about two standard deviations or more above the mean normal value. However, essentially all clinical diagnostic tests produce some percentage of false positives and false negatives. The sensitivity and selectivity of the diagnostic assay must be sufficient to satisfy the diagnostic objective and any relevant regulatory requirements. In general, the diagnostic methods of the invention are used to identify individuals as disease candidates, providing an additional parameter in a differential diagnosis of disease made by a competent health professional.

2. NF-AT_c Polynucleotides

Genomic or cDNA clones encoding NF-AT_c may be isolated from clone libraries (e.g., available from Clontech, Palo Alto, CA) using hybridization probes designed on the basis of the nucleotide sequences shown in Fig. 12 and using conventional hybridization screening methods (e.g., Benton WD and Davis RW (1977) *Science* 196: 180; Goodspeed et al. (1989) *Gene* 76: 1; Dunn et al. (1989) *J. Biol. Chem.* 264: 13057). Where a cDNA clone is desired, clone libraries containing cDNA derived from T cell mRNA is preferred. Alternatively, synthetic polynucleotide sequences corresponding to all or part of the sequences shown in Fig. 12 may be constructed by chemical synthesis of oligonucleotides. Additionally, polymerase chain reaction (PCR) using primers based on the sequence data disclosed in Fig. 12 may be used to amplify DNA fragments from genomic DNA, mRNA pools, or from cDNA clone libraries. U.S. Patents 4,683,195 and 4,683,202 describe the PCR method. Additionally, PCR methods employing one primer that is based on the sequence data disclosed in Fig. 12 and a second primer that is not based on that sequence data may be used. For example, a second primer that is homologous to or complementary to a polyadenylation segment may be used. In an embodiment, a polynucleotide comprising the 2742 nucleotide-long sequence of Fig. 12 can be used. Alternative polynucleotides encoding the 716 amino acid sequence of Fig. 12 can also be readily constructed by those of skill

in the art by using the degeneracy of the genetic code. Polynucleotides encoding amino acids 418 to 710 of the NF-AT_c sequence of Fig. 12 can also be constructed by those of skill in the art.

It is apparent to one of skill in the art that nucleotide substitutions, deletions, and additions may be incorporated into the polynucleotides of the invention. Nucleotide sequence variation may result from sequence polymorphisms of various NF-AT_c alleles, minor sequencing errors, and the like. However, such nucleotide substitutions, deletions, and additions should not substantially disrupt the ability of the polynucleotide to hybridize to one of the polynucleotide sequences shown in Fig. 12 under hybridization conditions that are sufficiently stringent to result in specific hybridization.

Specific hybridization is defined herein as the formation of hybrids between a probe polynucleotide (e.g., a polynucleotide of the invention which may include substitutions, deletion, and/or additions) and a specific target polynucleotide (e.g., a polynucleotide having the sequence in Fig. 12), wherein the probe preferentially hybridizes to the specific target such that, for example, a single band corresponding to NF-AT_c mRNA (or bands corresponding to multiple alternative splicing products of the NF-AT_c gene) can be identified on a Northern blot of RNA prepared from a suitable cell source (e.g., a T cell expressing NF-AT_c). Polynucleotides of the invention and recombinantly produced NF-AT_c, and fragments or analogs thereof, may be prepared on the basis of the sequence data provided in Fig. 12 according to methods known in the art and described in Maniatis et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed., (1989), Cold Spring Harbor, N.Y. and Berger and Kimmel, Methods in Enzymology, Volume 152, *Guide to Molecular Cloning Techniques* (1987), Academic Press, Inc., San Diego, CA, which are incorporated herein by reference.

NF-AT_c polynucleotides may be short oligonucleotides (e.g., 25-100 bases long), such as for use as hybridization probes and PCR (or LCR) primers. NF-AT_c polynucleotide sequences may also comprise part of a larger polynucleotide (e.g., a cloning vector comprising a NF-AT_c clone) and may be fused, by polynucleotide linkage, in frame with another polynucleotide sequence encoding a different protein (e.g., glutathione S-transferase or β-galactosidase) for encoding expression of a fusion protein. Typically, NF-AT_c polynucleotides comprise at least 25 consecutive nucleotides which are substantially identical to a naturally-occurring NF-AT_c sequence (e.g., Fig. 12), more usually NF-AT_c polynucleotides comprise at least 50 to 100 consecutive nucleotides which are substantially identical to a naturally-occurring NF-AT_c sequence. However, it will be recognized by those of skill that the minimum length of a NF-AT_c polynucleotide required for specific hybridization to a NF-AT_c target sequence will depend on several factors: G/C content, positioning of mismatched bases (if any), degree of uniqueness of the sequence as compared to the

population of target polynucleotides, and chemical nature of the polynucleotide (e.g., methylphosphonate backbone, phosphorothiolate, etc.), among others.

For example but not limitation, suitable hybridization probes for detecting and/or quantifying the presence of NF-AT$_c$ mRNA in a sample generally comprise at least one, preferably at least two, and more preferably all of the following human NF-AT$_c$ sequences shown in Table I, or their complements:

Table I: Selected Human NF-AT$_c$ Polynucleotide Sequences

5'-TTC CTC CGG GGC GCG CGG CGT GAG CCC GGG GCG AGG-3' (SEQ ID NO: 1);
5'-CAG CGC GGG GCG GCC ACT TCT CCT GTG CCT CCG CCC GCT GCT-3' (SEQ ID NO: 2);
5'-GCC GCG CGG ATG CCA AGC ACC AGC TTT CCA GTC CCT TCC AAG-3' (SEQ ID NO: 3);
5'-CCA ACG TCA GCC CCG CCC TGC CGC TCC CCA CGG CGC ACT CCA-3' (SEQ ID NO: 4);
5'-TTC AGA CCT CCA CAC CGG GCA TCA TCC CGC CGG CGG-3' (SEQ ID NO: 5);
5'-GCC ACA CCA GGC CTG ATG GGG CCC CTG CCC TGG AGA GTC CTC-3' (SEQ ID NO: 6);
5'-AGT CTG CCC AGC CTG GAG GCC TAC AGA GAC CCC TCG TGC CTG-3' (SEQ ID NO: 7);
5'-GTG TCT CCC AAG ACC ACG GAC CCC GAG GAG GGC TTT CCC-3' (SEQ ID NO: 8);
5'-AGC TGG CTG GGT GCC CGC TCC TCC AGA CCC GCG TCC CCT TGC-3' (SEQ ID NO: 9);
5'-TAC AGC CTC AAC GGC CGG CAG CCG CCC TAC TCA CCC CAC CAC-3' (SEQ ID NO: 10);
5'-GAC CAC CGA CAG CAG CCT GGA CCT GGG AGA TGG CGT CCC TGT-3' (SEQ ID NO: 11);
5'-CCT GGG CAG CCC CCC GCC CCC GGC CGA CTT CGC GCC CGA AGA-3' (SEQ ID NO: 12);
5'-GCT CCC CTA CCA GTG GCG AAG CCC AAG CCC CTG TCC CCT ACG-3' (SEQ ID NO: 13);
5'-CTT CGG ATT GAG GTG CAG CCC AAG TCC CAC CAC CGA GCC CAC-3' (SEQ ID NO: 14);
5'-CAT GGC TAC TTG GAG AAT GAG CCG CTG ATG CTG CAG CTT TTC-3' (SEQ ID NO: 15);
5'-AAG ACC GTG TCC ACC ACC AGC CAC GAG GCT ATC CTC TCC AAC-3' (SEQ ID NO: 16);
5'-TCA GCT CAG GAG CTG CCT CTG GTG GAG AAG CAG AGC ACG GAC-3' (SEQ ID NO: 17);
5'-AAC GCC ATC TTT CTA ACC GTA AGC CGT GAA CAT GAG CGC G-3' (SEQ ID NO: 18);
5'-AGA AAC GAC GTC GCC GTA AAG CAG CGT GGC GTG TGG CA-3' (SEQ ID NO: 19); and
5'-GCA TAC TCA GAT AGT CAC GGT TAT TTT GCT TCT TGC GAA TG-3' (SEQ ID NO: 20).

Also for example but not limitation, the following pair of PCR primers (amplimers) may be used to amplify murine or human NF-AT$_c$ sequences (e.g., by reverse transcriptase initiated PCR of RNA from NF-AT$_c$ expressing cells):

(forward) 5'-AGGGCGCGGGCACCGGGGCGCGGGCAGGGCTCGGAG-3' (SEQ ID NO: 21)
(reverse) 5'-GCAAGAAGCAAAATAACCGTGACTATCTGAGTATGC-3' (SEQ ID NO: 22)

If desired, PCR amplimers for amplifying substantially full-length cDNA copies may be selected at the discretion of the practioner. Similarly, amplimers to amplify single NF-AT_c exons or portions of the NF-AT_c gene (murine or human) may be selected.

Each of these sequences may be used as hybridization probes or PCR amplimers to detect the presence of NF-AT_c mRNA, for example to diagnose a disease characterized by the presence of an elevated NF-AT_c mRNA level in lymphocytes, or to perform tissue typing (i.e., identify tissues characterized by the expression of NF-AT_c mRNA), and the like. The sequences may also be used for detecting genomic NF-AT_c gene sequences in a DNA sample, such as for forensic DNA analysis (e.g., by RFLP analysis, PCR product length(s) distribution, etc.) or for diagnosis of diseases characterized by amplification and/or rearrangements of the NF-AT_c gene.

Disclosure of the full coding sequence for human NF-AT_c shown in Fig. 12 makes possible the construction of isolated polynucleotides that can direct the expression of NF-AT_c, fragments thereof, or analogs thereof. Further, the sequences in Fig. 12 make possible the construction of nucleic acid hybridization probes and PCR primers that can be used to detect RNA and DNA sequences encoding NF-AT_c.

Polynucleotides encoding full-length NF-AT_c or fragments or analogs thereof, may include sequences that facilitate transcription (expression sequences) and translation of the coding sequences, such that the encoded polypeptide product is produced. Construction of such polynucleotides is well known in the art and is described further in Maniatis et al., *Molecular Cloning: A Laboratory Manual*, 2nd Ed. (1989), Cold Spring Harbor, N.Y. For example, but not for limitation, such polynucleotides can include a promoter, a transcription termination site (polyadenylation site in eukaryotic expression hosts), a ribosome binding site, and, optionally, an enhancer for use in eukaryotic expression hosts, and, optionally, sequences necessary for replication of a vector. A typical eukaryotic expression cassette will include a polynucleotide sequence encoding a NF-AT_c polypeptide linked downstream (i.e., in translational reading frame orientation; polynucleotide linkage) of a promoter such as the HSV *tk* promoter or the *pgk* (phosphoglycerate kinase) promoter, optionally linked to an enhancer and a downstream polyadenylation site (e.g., an SV40 large T Ag poly A addition site).

A preferred NF-AT_c polynucleotide encodes a NF-AT_c polypeptide that comprises at least one of the following amino acids sequences:

-NAIFLTVSREHERVGC- (SEQ ID NO: 25);
-LHGYLENEPLMLQLFIGT- (SEQ ID NO: 26);
-PSTSPRASVTEESWLG- (SEQ ID NO: 27);
-GPAPRAGGTMKSAEEEHYG- (SEQ ID NO: 28);

-ASAGGHPIVQ- (SEQ ID NO: 29);
-NTRVRLVFRV- (SEQ ID NO: 30);
-AKTDRDLCKPNSLVVEIPPFRN- (SEQ ID NO: 31);
-EVQPKSHHRAHYETEGSR- (SEQ ID NO: 32);
-SPRVSVTDDSWLGNT- (SEQ ID NO: 33);
-SHHRAHYETEGSRGAV- (SEQ ID NO: 34);
-LRNSDIELRKGETDIGR- (SEQ ID NO: 35); and
-TLSLQVASNPIEC- (SEQ ID NO: 36).

The degeneracy of the genetic code gives a finite set of polynucleotide sequences encoding these amino acid sequences; this set of degenerate sequences may be readily generated by hand or by computer using commercially available software (Wisconsin Genetics Software Package Relaes 7.0). Thus, isolated polynucleotides typically less than approximately 10,000 nucleotides in length and comprising sequences encoding each of the following amino acid sequences:

-NAIFLTVSREHERVGC- (SEQ ID NO: 25);
-LHGYLENEPLMLQLFIGT- (SEQ ID NO: 26);
-PSTSPRASVTEESWLG- (SEQ ID NO: 27);
-GPAPRAGGTMKSAEEEHYG- (SEQ ID NO: 28);
-ASAGGHPIVQ- (SEQ ID NO: 29);
-NTRVRLVFRV- (SEQ ID NO: 30);
-AKTDRDLCKPNSLVVEIPPFRN- (SEQ ID NO: 31);
-EVQPKSHHRAHYETEGSR- (SEQ ID NO: 32);
-SPRVSVTDDSWLGNT- (SEQ ID NO: 33);
-SHHRAHYETEGSRGAV- (SEQ ID NO: 34);
-LRNSDIELRKGETDIGR- (SEQ ID NO: 35); and
-TLSLQVASNPIEC- (SEQ ID NO: 36).

are provided and may be used for, among other uses, the expression of a NF-AT_c polypeptide which can be used as an immunogen, immunological reagent, and the like. Such polynucleotides typically comprise an operably linked promoter for driving expression in a suitable prokaryotic or eukaryotic host cell. One exemplification of such a polynucleotide is the human NF-AT_c cDNA sequence of Fig. 12 cloned in operable linkage to the mammalian expression vector pSRα, many alternative embodiments will be apparent to those of skill in the art, including the use of alternative expression vectors (e.g., pBC12BI and p91023(B); Hanahan J (1983) *J. Mol. Biol.* 166: 577; Cullen et al. (1985) *J. Virol.* 53: 515; Lomedico PT (1982) *Proc. Natl. Acad. Sci. (U.S.A.)* 79: 5798; Morinaga et al. (1984) *Bio/Technology* 2: 636).

Additionally, where expression of a polypeptide is not desired, polynucleotides of this invention need not encode a functional protein. Polynucleotides of this invention may serve as hybridization probes and/or PCR primers (amplimers) and/or LCR oligomers for detecting NF-AT_c RNA or DNA sequences.

Alternatively, polynucleotides of this invention may serve as hybridization probes or primers for detecting RNA or DNA sequences of related genes, such genes may encode structurally or evolutionarily related proteins. For such hybridization and PCR applications, the polynucleotides of the invention need not encode a functional polypeptide. Thus, polynucleotides of the invention may contain substantial deletions, additions, nucleotide substitutions and/or transpositions, so long as specific hybridization or specific amplification to the NF-AT_c sequence is retained.

Specific hybridization is defined hereinbefore, and can be roughly summarized as the formation of hybrids between a polynucleotide of the invention (which may include substitutions, deletions, and/or additions) and a specific target polynucleotide such as human NF-AT_c mRNA so that a single band is identified corresponding to each NF-AT_c isoform on a Northern blot of RNA prepared from T cells (i.e., hybridization and washing conditions can be established that permit detection of discrete NF-AT_c mRNA band(s)). Thus, those of ordinary skill in the art can prepare polynucleotides of the invention, which may include substantial additions, deletions, substitutions, or transpositions of nucleotide sequence as compared to sequences shown in Fig. 12 and determine whether specific hybridization is a property of the polynucleotide by performing a Northern blot using RNA prepared from a T lymphocyte cell line which expresses NF-AT_c mRNA and/or by hybridization to a NF-AT_c DNA clone (cDNA or genomic clone).

Specific amplification is defined as the ability of a set of PCR amplimers, when used together in a PCR reaction with a NF-AT_c polynucleotide, to produce substantially a single major amplification product which corresponds to a NF-AT_c gene sequence or mRNA sequence. Generally, human genomic DNA or mRNA from NF-AT_c expressing human cells (e.g., Jurkat cell line) is used as the template DNA sample for the PCR reaction. PCR amplimers that exhibit specific amplification are suitable for quantitative determination of NF-AT_c mRNA by quantitative PCR amplification. NF-AT_c allele-specific amplification products, although having sequence and/or length polymorphisms, are considered to constitute a single amplification product for purposes of this definition.

Generally, hybridization probes comprise approximately at least 25 consecutive nucleotides of a sequence shown in Fig. 12 (for human and murine NF-AT_c detection, respectively), preferably the hybridization probes contain at least 50 consecutive nucleotides of a sequence shown in Fig. 12, and more preferably comprise at least 100 consecutive nucleotides of a sequence shown in Fig. 12. PCR amplimers typically comprise approximately 25 to 50 consecutive nucleotides of a

sequence shown in Fig. 12, and usually consist essentially of approximately 25 to 50 consecutive nucleotides of a sequence shown in Fig. 12 with additional nucleotides, if present, generally being at the 5' end so as not to interfere with polymerase-mediated chain extension. PCR amplimer design and hybridization probe selection are well within the scope of discretion of practioners of ordinary skill in the art.

In one preferred embodiment of the invention, hybridization probes that specifically identify the NF-AT$_c$ gene may be used in methods for diagnosing genetic disease. For example, but not for limitation, the genetic disease thus diagnosed may involve a lesion in the relevant NF-AT$_c$ structural or regulatory sequences, or may involve a lesion in a genetic locus closely linked to the NF-AT$_c$ locus and which can be identified by restriction fragment length polymorphism or DNA sequence polymorphism at the linked NF-AT$_c$ locus. In a further preferred embodiment, NF-AT$_c$ gene probes are used to diagnose or identify genetic disease involving predisposition to immunological disease, wherein the amount or functionality of endogenous NF-AT$_c$ is sufficient for the individual to exhibit an increased probability of developing an immune disease, particularly an immune deficiency, arthritis, or autoimmune disease.

3. Isolation of the Cognate Human NF-AT$_c$ Gene

The human homolog of the NF-AT$_c$ cDNA is identified and isolated by screening a human genomic clone library, such as a human genomic library in yeast artificial chromosomes, cosmids, or bacteriophage λ (e.g., λ Charon 35), with a polynucleotide probe comprising a sequence of about at least 24 contiguous nucleotides (or their complement) of the cDNA sequence shown in Fig. 12. Typically, hybridization and washing conditions are performed at high stringency according to conventional hybridization procedures. Positive clones are isolated and sequenced. For illustration and not for limitation, a full-length polynucleotide corresponding to the sequence of Fig. 12 may be labeled and used as a hybridization probe to isolate genomic clones from a human or murine genomic clone libary in λEMBL4 or λGEM11 (Promega Corporation, Madison, Wisconsin); typical hybridization conditions for screening plaque lifts (Benton and Davis (1978) *Science* 196: 180) can be: 50% formamide, 5 x SSC or SSPE, 1-5 x Denhardt's solution, 0.1-1% SDS, 100-200 μg sheared heterologous DNA or tRNA, 0-10% dextran sulfate, 1 x10^5 to 1 x 10^7 cpm/ml of denatured probe with a specific activity of about 1 x 10^8 cpm/μg, and incubation at 42°C for about 6-36 hours. Prehybridization conditions are essentially identical except that probe is not included and incubation time is typically reduced. Washing conditions are typically 1-3 x SSC, 0.1-1% SDS, 50-70°C with change of wash solution at about 5-30 minutes.

Nonhuman NF-AT$_c$ cDNAs and genomic clones (i.e., cognate nonhuman NF-AT$_c$ genes) can be analogously isolated from various nonhuman cDNA and genomic clone libraries available

in the art (e.g., Clontech, Palo Alto, CA) by using probes based on the sequences shown in Fig. 12, with hybridization and washing conditions typically being less stringent than for isolation of human NF-AT$_c$ clones.

Polynucleotides comprising sequences of approximately at least 30-50 nucleotides, preferably at least 100 nucleotides, corresponding to or complementary to the nucleotide sequences shown in Fig. 12 can serve as PCR primers and/or hybridization probes for identifying and isolating germline genes corresponding to NF-AT$_c$. These germline genes may be human or may be from a related mammalian species, preferably rodents or primates. Such germline genes may be isolated by various methods conventional in the art, including, but not limited to, by hybridization screening of genomic libraries in bacteriophage λ or cosmid libraries, or by PCR amplification of genomic sequences using primers derived from the sequences shown in Fig. 12. Human genomic libraries are publicly available or may be constructed de novo from human DNA.

Genomic clones of NF-AT$_c$, particularly of the murine cognate NF-AT gene, may be used to construct homologous targeting constructs for generating cells and transgenic nonhuman animals having at least one functionally disrupted NF-AT$_c$ allele, preferably homozygous for knocked out NF-AT$_c$ alleles. Guidance for construction of homologous targeting constructs may be found in the art, including: Rahemtulla et al. (1991) *Nature* 353: 180; Jasin et al. (1990) *Genes Devel.* 4: 157; Koh et al. (1992) *Science* 256: 1210; Molina et al. (1992) *Nature* 357: 161; Grusby et al. (1991) *Science* 253: 1417; Bradley et al. (1992) *Bio/Technology* 10: 534, incorporated herein by reference). Homologous targeting can be used to generate so-called "knockout" mice, which are heterozygous or homozygous for an inactivated NF-AT$_c$ allele. Such mice may be sold commercially as research animals for investigation of immune system development, neoplasia, T cell activation, signal transduction, drug sreening, and other uses.

Chimeric targeted mice are derived according to Hogan, et al., *Manipulating the Mouse Embryo: A Laboratory Manual*, Cold Spring Harbor Laboratory (1988) and *Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed., IRL Press, Washington, D.C., (1987) which are incorporated herein by reference. Embryonic stem cells are manipulated according to published procedures (*Teratocarcinomas and Embryonic Stem Cells: A Practical Approach*, E.J. Robertson, ed., IRL Press, Washington, D.C. (1987); Zjilstra et al. (1989) *Nature* 342:435; and Schwartzberg et al. (1989) *Science* 246: 799, each of which is incorporated herein by reference).

Additionally, a NF-AT$_c$ cDNA or genomic gene copy may be used to construct transgenes for expressing NF-AT$_c$ polypeptides at high levels and/or under the transcriptional control of transcription control sequences which do not naturally occur adjacent to the NF-AT$_c$ gene. For example but not limitation, a constitutive promoter (e.g., a HSV-*tk* or *pgk* promoter) or a cell-

lineage specific transcriptional regulatory sequence (e.g., a CD4 or CD8 gene promoter/enhancer) may be operably linked to a NF-AT_c-encoding polynucleotide sequence to form a transgene (typically in combination with a selectable marker such as a *neo* gene expression cassette). Such transgenes can be introduced into cells (e.g., ES cells, hematopoietic stem cells) and transgenic cells and transgenic nonhuman animals may be obtained according to conventional methods. Transgenic cells and/or transgenic nonhuman animals may be used to screen for antineoplastic agents and/or to screen for potential immunomodulatory agents, as overexpression of NF-AT_c or inappropriate expression of NF-AT_c may result in a hyperimmune state or enhance graft rejection reactions.

4. Antisense Polynucleotides

Additional embodiments directed to modulation of T cell activation include methods that employ specific antisense polynucleotides complementary to all or part of the sequences shown in Fig. 12. Such complementary antisense polynucleotides may include nucleotide substitutions, additions, deletions, or transpositions, so long as specific hybridization to the relevant target sequence corresponding to Fig. 12 is retained as a functional property of the polynucleotide. Complementary antisense polynucleotides include soluble antisense RNA or DNA oligonucleotides which can hybridize specifically to NF-AT_c mRNA species and prevent transcription of the mRNA species and/or translation of the encoded polypeptide (Ching et al. (1989) *Proc. Natl. Acad. Sci. U.S.A.* 86: 10006; Broder et al. (1990) *Ann. Int. Med.* 113: 604; Loreau et al. (1990) *FEBS Letters* 274: 53; Holcenberg et al., WO91/11535; U.S.S.N. 07/530,165; WO91/09865; WO91/04753; WO90/13641; and EP 386563, each of which is incorporated herein by reference). The antisense polynucleotides therefore inhibit production of NF-AT_c polypeptides. Since NF-AT_c protein expression is associated with T lymphocyte activation, antisense polynucleotides that prevent transcription and/or translation of mRNA corresponding to NF-AT_c polypeptides may inhibit T cell activation and/or reverse the activated phenotype of T cells. Compositions containing a therapeutically effective dosage of NF-AT_c antisense polynucleotides may be administered for treatment of immune diseases, including lymphocytic leukemias, and for inhibition of transplant rejection reactions, if desired. Antisense polynucleotides of various lengths may be produced, although such antisense polynucleotides typically comprise a sequence of about at least 25 consecutive nucleotides which are substantially identical to a naturally-occurring NF-AT_c polynucleotide sequence, and typically which are identical to a sequence shown in Fig. 12.

Antisense polynucleotides may be produced from a heterologous expression cassette in a transfectant cell or transgenic cell, such as a transgenic pluripotent hematopoietic stem cell used to reconstitute all or part of the hematopoietic stem cell population of an individual. Alternatively,

the antisense polynucleotides may comprise soluble oligonucleotides that are administered to the external milieu, either in the culture medium *in vitro* or in the circulatory system or interstitial fluid *in vivo*. Soluble antisense polynucleotides present in the external milieu have been shown to gain access to the cytoplasm and inhibit translation of specific mRNA species. In some embodiments the antisense polynucleotides comprise methylphosphonate moieties. For general methods relating to antisense polynucleotides, see *Antisense RNA and DNA*, (1988), D.A. Melton, Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY).

5. NF-AT$_c$ Polypeptides

The nucleotide and amino acid sequences shown in Fig. 12 enable those of skill in the art to produce polypeptides corresponding to all or part of the full-length human NF-AT$_c$ polypeptide sequence. Such polypeptides may be produced in prokaryotic or eukaryotic host cells by expression of polynucleotides encoding NF-AT$_c$, or fragments and analogs thereof. Alternatively, such polypeptides may be synthesized by chemical methods or produced by *in vitro* translation systems using a polynucleotide template to direct translation. Methods for expression of heterologous proteins in recombinant hosts, chemical synthesis of polypeptides, and *in vitro* translation are well known in the art and are described further in Maniatis et al., *Molecular Cloning: A Laboratory Manual* (1989), 2nd Ed., Cold Spring Harbor, N.Y. and Berger and Kimmel, *Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques* (1987), Academic Press, Inc., San Diego, CA.

Fragments or analogs of NF-AT$_c$ may be prepared by those of skill in the art. Preferred amino- and carboxy-termini of fragments or analogs of NF-AT$_c$ occur near boundaries of functional domains. For example, but not for limitation, such functional domains include: (1) domains conferring the property of binding to other NF-AT components (e.g., AP-1), (2) domains conferring the property of nuclear localization in stimulated T lymphocytes, and (3) domains conferring the property of enhancing activation of T cells when expressed at sufficient levels in such cells. Additionally, such functional domains might include: (1) domains conferring the property of binding to RNA polymerase species, (2) domains having the capacity to directly alter local chromatin structure, which may comprise catalytic activities (e.g., topoisomerases, endonucleases) and/or which may comprise structural features (e.g., zinc fingers, histone-binding moieties), and (3) domains which may interact with accessory proteins and/or transcription factors.

One method by which structural and functional domains may be identified is by comparison of the nucleotide and/or amino acid sequence data shown in Fig. 12 to public or proprietary sequence databases. Preferably, computerized comparison methods are used to identify sequence motifs or predicted protein conformation domains that occur in other proteins of known structure

and/or function, such as the zinc fingers. For example, the NAD-binding domains of dehydrogenases, particularly lactate dehydrogenase and malate dehydrogenase, are similar in conformation and have amino acid sequences that are detectably homologous (*Proteins, Structures and Molecular Principles*, (1984) Creighton (ed.), W.H. Freeman and Company, New York, which is incorporated herein by reference). Further, a method to identify protein sequences that fold into a known three-dimensional structure are known (Bowie et al. (1991) *Science* 253: 164). Thus, the foregoing examples demonstrate that those of skill in the art can recognize sequence motifs and structural conformations that may be used to define structural and functional domains in the NF-AT$_c$ sequences of the invention. One example of a domain is the *rel* similarity region from amino acid 418 to amino acid 710 of the NF-AT$_c$ polypeptide sequence of Fig. 12.

Additionally, computerized comparison of sequences shown in Fig. 12 to existing sequence databases can identify sequence motifs and structural conformations found in other proteins or coding sequences that indicate similar domains of the NF-AT$_c$ protein. For example but not for limitation, the programs GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package (Genetics Computer Group, 575 Science Dr., Madison, WI) can be used to identify sequences in databases, such as GenBank/EMBL, that have regions of homology with a NF-AT$_c$ sequences. Such homologous regions are candidate structural or functional domains. Alternatively, other algorithms are provided for identifying such domains from sequence data. Further, neural network methods, whether implemented in hardware or software, may be used to: (1) identify related protein sequences and nucleotide sequences, and (2) define structural or functional domains in NF-AT$_c$ polypeptides (Brunak et al. (1991) *J. Mol. Biol.* 220: 49, which is incorporated herein by reference). For example, the 13-residue repeat motifs -SPRASVTEESWLG- (SEQ ID NO: 23) and -SPRVSVTDDSWLG-(SEQ ID NO: 24) are examples of structurally related domains.

Fragments or analogs comprising substantially one or more functional domain may be fused to heterologous polypeptide sequences, wherein the resultant fusion protein exhibits the functional property(ies) conferred by the NF-AT$_c$ fragment. Alternatively, NF-AT$_c$ polypeptides wherein one or more functional domain have been deleted will exhibit a loss of the property normally conferred by the missing fragment.

By way of example and not limitation, the domain conferring the property of nuclear localization and/or interaction with AP-1 may be fused to β-galactosidase to produce a fusion protein that is localized to the nucleus and which can enzymatically convert a chromogenic substrate to a chromophore.

Although one class of preferred embodiments are fragments having amino- and/or carboxy-termini corresponding to amino acid positions near functional domains borders, alternative NF-AT$_c$

fragments may be prepared. The choice of the amino- and carboxy-termini of such fragments rests with the discretion of the practitioner and will be made based on experimental considerations such as ease of construction, stability to proteolysis, thermal stability, immunological reactivity, amino- or carboxyl-terminal residue modification, or other considerations.

In addition to fragments, analogs of NF-AT$_c$ can be made. Such analogs may include one or more deletions or additions of amino acid sequence, either at the amino- or carboxy-termini, or internally, or both; analogs may further include sequence transpositions. Analogs may also comprise amino acid substitutions, preferably conservative substitutions. Additionally, analogs may include heterologous sequences generally linked at the amino- or carboxy-terminus, wherein the heterologous sequence(s) confer a functional property to the resultant analog which is not indigenous to the native NF-AT$_c$ protein. However, NF-AT$_c$ analogs must comprise a segment of 25 amino acids that has substantial similarity to a portion of the amino acid sequence shown in Fig. 12, respectively, and which has at least one of the requisite functional properties enumerated in the Definitions (*supra*). Preferred amino acid substitutions are those which: (1) reduce susceptibility to proteolysis, (2) reduce susceptibility to oxidation, (3) alter post-translational modification of the analog, possibly including phosphorylation, and (4) confer or modify other physicochemical or functional properties of such analogs, possibly including interaction with calcineurin or phophorylation or dephosphorylation thereby. NF-AT$_c$ analogs include various muteins of a NF-AT$_c$ sequence other than the naturally-occurring peptide sequence. For example, single or multiple amino acid substitutions (preferably conservative amino acid substitutions) may be made in the naturally-occurring NF-AT$_c$ sequence (preferably in the portion of the polypeptide outside the functional domains).

Conservative amino acid substitution is a substitution of an amino acid by a replacement amino acid which has similar characteristics (e.g., those with acidic properties: Asp and Glu). A conservative (or synonymous) amino acid substitution should not substantially change the structural characteristics of the parent sequence (e.g., a replacement amino acid should not tend to break a helix that occurs in the parent sequence, or disrupt other types of secondary structure that characterizes the parent sequence). Examples of art-recognized polypeptide secondary and tertiary structures are described in *Proteins, Structures and Molecular Principles*, (1984) Creighton (ed.), W.H. Freeman and Company, New York; *Introduction to Protein Structure*, (1991), C. Branden and J. Tooze, Garland Publishing, New York, NY; and Thornton et al. (1991) *Nature* 354: 105; which are incorporated herein by reference).

Native NF-AT$_c$ proteins, fragments thereof, or analogs thereof can be used as reagents in DNA binding assays and/or *in vitro* transcription assays for identifying agents that interfere with NF-AT function, said agents are thereby identified as candidate drugs which may be used, for

example, to block T cell activation or treat T cell lymphocytic leukemias. Typically, *in vitro* DNA binding assays that measure binding of NF-AT to DNA employ double-stranded DNA that contains an array of one or more NF-AT recognition sites (as defined by specific footprinting of native NF-AT protein). The DNA is typically linked to a solid substrate by any of various means known to those of skill in the art; such linkage may be noncovalent (e.g., binding to a highly charged surface such as Nylon 66) or may be by covalent bonding (e.g., typically by chemical linkage involving a nitrogen position in a nucleotide base, such as diazotization). NF-AT_c polypeptides are typically labeled by incorporation of a radiolabeled amino acid. The labeled NF-AT_c polypeptide, usually reconstituted with an NF-AT nuclear component (e.g., AP-1 activity) to form an NF-AT complex, is contacted with the immobilized DNA under aqueous conditions that permit specific binding in control binding reactions with a binding affinity of about 1 x 10^6 M^{-1} or greater (e.g., 10-250 mM NaCl or KCl and 5-100 mM Tris HCl pH 5-9, usually pH 6-8), generally including Zn^{+2} and/or Mn^{+2} and/or Mg^{+2} in the nanomolar to micromolar range (1 nM to 999 μM). Specificity of binding is typically established by adding unlabeled competitor at various concentrations selected at the discretion of the practitioner. Examples of unlabeled protein competitors include, but are not limited to, the following: unlabeled NF-AT_c polypeptide, bovine serum albumin, and nuclear protein extracts. Binding reactions wherein one or more agents are added are performed in parallel with a control binding reaction that does not include an agent. Agents which inhibit the specific binding of NF-AT_c polypeptides to DNA, as compared to a control reaction, are identified as candidate immunomodulatory drugs. Also, agents which prevent transcriptional modulation by NF-AT *in vitro* are thereby identified as candidate immunomodulatory drugs.

In addition to NF-AT_c polypeptides consisting only of naturally-occuring amino acids, NF-AT_c peptidomimetics are also provided. Peptide analogs are commonly used in the pharmaceutical industry as non-peptide drugs with properties analogous to those of the template peptide. These types of non-peptide compound are termed "peptide mimetics" or "peptidomimetics" (Fauchere, J. (1986) *Adv. Drug Res.* 15: 29; Veber and Freidinger (1985) TINS p.392; and Evans et al. (1987) *J. Med. Chem* 30: 1229, which are incorporated herein by reference) and are usually developed with the aid of computerized molecular modeling. Peptide mimetics that are structurally similar to therapeutically useful peptides may be used to produce an equivalent therapeutic or prophylactic effect. Generally, peptidomimetics are structurally similar to a paradigm polypeptide (i.e., a polypeptide that has a biological or pharmacological activity), such as human NF-AT_c, but have one or more peptide linkages optionally replaced by a linkage selected from the group consisting of: -CH_2NH-, -CH_2S-, -CH_2-CH_2-, -CH=CH- (cis and trans), -$COCH_2$-, -$CH(OH)CH_2$-, and -CH_2SO-, by methods known in the art and further described in the following references: Spatola, A.F. in *"Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins,"* B.

Weinstein, eds., Marcel Dekker, New York, p. 267 (1983); Spatola, A.F., Vega Data (March 1983), Vol. 1, Issue 3, "Peptide Backbone Modifications" (general review); Morley, J.S., *Trends Pharm Sci* (1980) pp. 463-468 (general review); Hudson, D. et al., *Int J Pept Prot Res* (1979) 14:177-185 (-CH_2NH-, CH_2CH_2-); Spatola, A.F. et al., *Life Sci* (1986) 38:1243-1249 (-CH_2-S); Hann, M.M., *J Chem Soc Perkin Trans I* (1982) 307-314 (-CH-CH-, cis and trans); Almquist, R.G. et al., *J Med Chem* (1980) 23:1392-1398 (-$COCH_2$-); Jennings-White, C. et al., *Tetrahedron Lett* (1982) 23:2533 (-$COCH_2$-); Szelke, M. et al., European Appln. EP 45665 (1982) CA: 97:39405 (1982) (-$CH(OH)CH_2$-); Holladay, M.W. et al., *Tetrahedron Lett* (1983) 24:4401-4404 (-$C(OH)CH_2$-); and Hruby, V.J., Life Sci (1982) 31:189-199 (-CH_2-S-); each of which is incorporated herein by reference. A particularly preferred non-peptide linkage is -CH_2NH-. Such peptide mimetics may have significant advantages over polypeptide embodiments, including, for example: more economical production, greater chemical stability, enhanced pharmacological properties (half-life, absorption, potency, efficacy, etc.), altered specificity (e.g., a broad-spectrum of biological activities), reduced antigenicity, and others. Labeling of peptidomimetics usually involves covalent attachment of one or more labels, directly or through a spacer (e.g., an amide group), to non-interfering position(s) on the peptidomimetic that are predicted by quantitative structure-activity data and/or molecular modeling. Such non-interfering positions generally are positions that do not form direct contacts with the macromolecules(s) (e.g., immunoglobulin superfamily molecules) to which the peptidomimetic binds to produce the therapeutic effect. Derivitization (e.g., labelling) of peptidomimetics should not substantially interfere with the desired biological or pharmacological activity of the peptidomimetic. Peptidomimetics of NF-AT_c may be used as competitive or noncompetitive agonists or antagonists of NF-AT_c function. For example, a NF-AT_c peptidomimetic administered to a stimulated T cell containing NF-AT_c and may compete with the naturally-occurring NF-AT_c and reduce NF-AT activity. Alternatively, an NF-AT_c peptidomimetic administerd to a T cell lacking NF-AT_c may induce T cell activation or the like.

Systematic substitution of one or more amino acids of a consensus sequence with a D-amino acid of the same type (e.g., D-lysine in place of L-lysine) may be used to generate more stable peptides. In addition, constrained peptides (including cyclized peptides) comprising a consensus sequence or a substantially identical consensus sequence variation may be generated by methods known in the art (Rizo and Gierasch (1992) *Ann. Rev. Biochem.* 61: 387, incorporated herein by reference); for example, by adding internal cysteine residues capable of forming intramolecular disulfide bridges which cyclize the peptide.

The amino acid sequences of NF-AT_c polypeptides identified herein will enable those of skill in the art to produce polypeptides corresponding to NF-AT_c peptide sequences and sequence variants thereof. Such polypeptides may be produced in prokaryotic or eukaryotic host cells by

expression of polynucleotides encoding a NF-AT_c peptide sequence, frequently as part of a larger polypeptide. Alternatively, such peptides may be synthesized by chemical methods. Methods for expression of heterologous proteins in recombinant hosts, chemical synthesis of polypeptides, and *in vitro* translation are well known in the art and are described further in Maniatis et al., *Molecular Cloning: A Laboratory Manual* (1989), 2nd Ed., Cold Spring Harbor, N.Y.; Berger and Kimmel, *Methods in Enzymology*, Volume 152, *Guide to Molecular Cloning Techniques* (1987), Academic Press, Inc., San Diego, CA; Merrifield, J. (1969) *J. Am. Chem. Soc.* 91: 501; Chaiken I.M. (1981) *CRC Crit. Rev. Biochem.* 11: 255; Kaiser et al.(1989) *Science* 243: 187; Merrifield, B. (1986) *Science* 232: 342; Kent, S.B.H. (1988) *Ann. Rev. Biochem.* 57: 957; and Offord, R.E. (1980) *Semisynthetic Proteins*, Wiley Publishing, which are incorporated herein by reference).

Recombinant NF-AT polypeptides can be produced as follows.

The nucleic acid sequences of the present invention capable of ultimately expressing the desired NF-AT_c polypeptides can be formed from a variety of different polynucleotides (genomic or cDNA, RNA, synthetic oligonucleotides, etc.) as well as by a variety of different techniques.

As stated previously, the DNA sequences will be expressed in hosts after the sequences have been operably linked to (i.e., positioned to ensure the functioning of) an expression control sequence. These expression vectors are typically replicable in the host organisms either as episomes or as an integral part of the host chromosomal DNA. Commonly, expression vectors will contain selection markers, e.g., tetracycline resistance or hygromycin resistance, to permit detection and/or selection of those cells transformed with the desired DNA sequences (see, e.g., U.S. Patent 4,704,362, which is incorporated herein by reference).

E. coli is one prokaryotic host useful particularly for cloning the DNA sequences of the present invention. Other microbial hosts suitable for use include bacilli, such as *Bacillus subtilis*, and other Enterobacteriaceae, such as *Salmonella*, *Serratia*, and various *Pseudomonas* species. In these prokaryotic hosts, one can also make expression vectors, which will typically contain expression control sequences compatible with the host cell (e.g., an origin of replication). In addition, any number of a variety of well-known promoters will be present, such as the lactose promoter system, a tryptophan (trp) promoter system, a beta-lactamase promoter system, or a promoter system from phage lambda. The promoters will typically control expression, optionally with an operator sequence, and have ribosome binding site sequences and the like, for initiating and completing transcription and translation.

Other microbes, such as yeast, may also be used for expression. *Saccharomyces* is a preferred host, with suitable vectors having expression control sequences, such as promoters, including 3-phosphoglycerate kinase or other glycolytic enzymes, and an origin of replication, termination sequences and the like as desired.

In addition to microorganisms, mammalian tissue cell culture may also be used to express and produce the polypeptides of the present invention (*see*, Winnacker, "*From Genes to Clones*," VCH Publishers, N.Y., N.Y. (1987), which is incorporated herein by reference). Eukaryotic cells are actually preferred, because a number of suitable host cell lines capable of secreting intact human proteins have been developed in the art, and include the CHO cell lines, various COS cell lines, HeLa cells, myeloma cell lines, Jurkat cells, etc. Expression vectors for these cells can include expression control sequences, such as an origin of replication, a promoter, an enhancer (Queen et al. (1986 *Immunol. Rev.* 89: 49, which is incorporated herein by reference), and necessary processing information sites, such as ribosome binding sites, RNA splice sites, polyadenylation sites, and transcriptional terminator sequences. Preferred expression control sequences are promoters derived from immunoglobulin genes, SV40, adenovirus, bovine papillomavirus, and the like. The vectors containing the DNA segments of interest (e.g., polypeptides encoding a NF-AT_c polypeptide) can be transferred into the host cell by well-known methods, which vary depending on the type of cellular host. For example, CaCl transfection is commonly utilized for prokaryotic cells, whereas $CaPO_4$ treatment or electroporation may be used for other cellular hosts. (See, generally, Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, (1982), which is incorporated herein by reference). Usually, vectors are episomes and are maintained extrachromosomally.

Expression of recombinant NF-ATc protein in cells, particularly cells of the lymphopoietic lineage, may be used to identify and isolate genes that are transcriptionally modulated, either positively or negatively, by the presence of NF-AT_c protein. Such genes are typically initially identified as cDNA clones isolated from subtractive cDNA libraries, wherein RNA isolated from cells expressing recombinant NF-AT and RNA isolated from control cells (i.e., not expressing recombinant NF-ATc) are used to generate the subtractive libraries and screening probes. In such a manner, NF-AT_c-dependent genes may be isolated. NFAT-dependent genes (or their regulatory sequences operably linked to a reporter gene) may be used as a component of an *in vitro* transcription assay employing a NF-AT_c polypeptide as a necessary component for efficient transcription; such transcription assays may be used to screen for agents which inhibit NF-AT_c-dependent gene transcription and are thereby identified as candidate immunomodulatory agents.

6. Production and Applications of α-NF-AT_c Antibodies

NF-AT_c and NF-ATn proteins, fragments thereof, or analogs thereof, may be used to immunize an animal for the production of specific antibodies. These antibodies may comprise a polyclonal antiserum or may comprise a monoclonal antibody produced by hybridoma cells. For general methods to prepare antibodies, see *Antibodies: A Laboratory Manual*, (1988) E. Harlow

and D. Lane, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, which is incorporated herein by reference.

For example but not for limitation, a recombinantly produced fragment of human NF-AT$_c$ or a purified NF-ATc protein can be injected into a rat or a mouse along with an adjuvant following immunization protocols known to those of skill in the art so as to generate an immune response. Typically, approximately at least 1-50 μg of a NF-AT$_c$ fragment or analog is used for the initial immunization, depending upon the length of the polypeptide. Alternatively or in combination with a recombinantly produced NF-AT$_c$ polypeptide, a chemically synthesized peptide having a NF-AT$_c$ sequence (e.g., peptides exemplified in Table II, *infra*) may be used as an immunogen to raise antibodies which bind a NF-AT$_c$ protein, such as the native human NF-AT$_c$ polypeptide having the sequence shown essentially in Fig. 12 or the native human NF-AT$_c$ polypeptide isoform. Immunoglobulins which bind the recombinant fragment with a binding affinity of at least 1 x 10^7 M^{-1} can be harvested from the immunized animal as an antiserum, and may be further purified by immunoaffinity chromatography or other means. Additionally, spleen cells are harvested from the immunized animal (typically rat or mouse) and fused to myeloma cells to produce a bank of antibody-secreting hybridoma cells. The bank of hybridomas can be screened for clones that secrete immunoglobulins which bind the recombinantly produced NF-AT$_c$ polypeptide (or chemically synthesized NF-AT$_c$ polypeptide) with an affinity of at least 1 x 10^6 M^{-1}. Animals other than mice and rats may be used to raise antibodies; for example, goats, rabbits, sheep, and chickens may also be employed to raise antibodies reactive with a NF-AT$_c$ protein. Transgenic mice having the capacity to produce substantially human antibodies also may be immunized and used for a source of α-NF-AT$_c$ antiserum and/or for making monoclonal-secreting hybridomas.

Bacteriophage antibody display libraries may also be screened for binding to a NF-AT$_c$ polypeptide, such as a full-length human NF-AT$_c$ protein, a NF-AT$_c$ fragment (e.g., a peptide having a sequence shown in Table II, *infra*), or a fusion protein comprising a NF-AT$_c$ polypeptide sequence of at least 14 contiguous amino acids as shown in Fig. 12 or a polypeptide sequence of Table II (*infra*). Combinatorial libraries of antibodies have been generated in bacteriophage lambda expression systems which may be screened as bacteriophage plaques or as colonies of lysogens (Huse et al. (1989) *Science* 246: 1275; Caton and Koprowski (1990) *Proc. Natl. Acad. Sci. (U.S.A.)* 87: 6450; Mullinax et al (1990) *Proc. Natl. Acad. Sci. (U.S.A.)* 87: 8095; Persson et al. (1991) *Proc. Natl. Acad. Sci. (U.S.A.)* 88: 2432). Various embodiments of bacteriophage antibody display libraries and lambda phage expression libraries have been described (Kang et al. (1991) *Proc. Natl. Acad. Sci. (U.S.A.)* 88: 4363; Clackson et al. (1991) *Nature* 352: 624; McCafferty et al. (1990) *Nature* 348: 552; Burton et al. (1991) *Proc. Natl. Acad. Sci. (U.S.A.)* 88: 10134;

Hoogenboom et al. (1991) *Nucleic Acids Res.* 19: 4133; Chang et al. (1991) *J. Immunol.* 147: 3610; Breitling et al. (1991) *Gene* 104: 147; Marks et al. (1991) *J. Mol. Biol.* 222: 581; Barbas et al. (1992) *Proc. Natl. Acad. Sci. (U.S.A.)* 89: 4457; Hawkins and Winter (1992) *J. Immunol.* 22: 867; Marks et al. (1992) *Biotechnology* 10: 779; Marks et al. (1992) *J. Biol. Chem.* 267: 16007; Lowman et al (1991) *Biochemistry* 30: 10832; Lerner et al. (1992) *Science* 258: 1313, incorporated herein by reference). Typically, a bacteriophage antibody display library is screened with a NF-AT$_c$ polypeptide that is immobilized (e.g., by covalent linkage to a chromatography resin to enrich for reactive phage by affinity chromatography) and/or labeled (e.g., to screen plaque or colony lifts).

NF-AT$_c$ polypeptides which are useful as immunogens, for diagnostic detection of α-NF-AT$_c$ antibodies in a sample, for diagnosic detection and quantitation of NF-AT$_c$ protein in a sample (e.g., by standardized competitive ELISA), or for screening a bacteriophage antibody display library, are suitably obtained in substantially pure form, that is, typically about 50 percent (w/w) or more purity, substantially free of interfering proteins and contaminants. Preferably, these polypeptides are isolated or synthesized in a purity of at least 80 percent (w/w) and, more preferably, in at least about 95 percent (w/w) purity, being substantially free of other proteins of humans, mice, or other contaminants. Preferred immunogens comprise at least one NF-AT$_c$ polypeptide sequence shown in Table II, either as a discrete peptide or as part of a fusion polypeptide (e.g., with a β-galactosidase or glutathione S-transferase sequence). NF-AT$_c$ immunogens comprise at least one, typically several of such immunogenic epitopes.

For some applications of these antibodies, such as identifying immunocrossreactive proteins, the desired antiserum or monoclonal antibody(ies) is/are not monospecific. In these instances, it may be preferable to use a synthetic or recombinant fragment of NF-AT$_c$ as an antigen rather than using the entire native protein. More specifically, where the object is to identify immunocrossreactive polypeptides that comprise a particular structural moiety, such as a DNA-binding domain, it is preferable to use as an antigen a fragment corresponding to part or all of a commensurate structural domain in the NF-AT$_c$ protein. Production of recombinant or synthetic fragments having such defined amino- and carboxy-termini is provided by the NF-AT$_c$ sequences shown in Fig. 12, may be accomplished by proteolytic digestion of a purified submit or produced recombinantly.

If an antiserum is raised to a NF-AT$_c$ fusion polypeptide, such as a fusion protein comprising a NF-AT$_c$ immunogenic epitope fused to β-galactosidase or glutathione S-transferase, the antiserum is preferably preadsorbed with the non-NF-AT$_c$ fusion partner (e.g, β-galactosidase or glutathione S-transferase) to deplete the antiserum of antibodies that react (i.e., specifically bind to) the non-NF-AT$_c$ portion of the fusion protein that serves as the immunogen. Monoclonal or

polyclonal antibodies which bind to the human and/or murine NF-AT_c protein can be used to detect the presence of human or murine NF-AT_c polypeptides in a sample, such as a Western blot of denatured protein (e.g., a nitrocellulose blot of an SDS-PAGE) obtained from a lymphocyte sample of a patient. Preferably quantitative detection is performed, such as by denistometric scanning and signal integration of a Western blot. The monoclonal or polyclonal antibodies will bind to the denatured NF-AT_c epitopes and may be identified visually or by other optical means with a labeled second antibody or labeled *Staphylococcus aureus* protein A by methods known in the art. Frequently, denatured NF-AT_c will be used as the target antigen so that more epitopes may be available for binding.

Table II

Selected Human NF-AT_c Antigen Peptides

-NAIFLTVSREHERVGC- (SEQ ID NO: 25);
-LHGYLENEPLMLQLFIGT- (SEQ ID NO: 26);
-PSTSPRASVTEESWLG- (SEQ ID NO: 27);
-GPAPRAGGTMKSAEEEHYG- (SEQ ID NO: 28);
-ASAGGHPIVQ- (SEQ ID NO: 29);
-NTRVRLVFRV- (SEQ ID NO: 30);
-AKTDRDLCKPNSLVVEIPPFRN- (SEQ ID NO: 31);
-EVQPKSHHRAHYETEGSR- (SEQ ID NO: 32);
-SPRVSVTDDSWLGNT- (SEQ ID NO: 33);
-SHHRAHYETEGSRGAV- (SEQ ID NO: 34);
-LRNSDIELRKGETDIGR- (SEQ ID NO: 35); and
-TLSLQVASNPIEC- (SEQ ID NO: 36).

Such NF-AT_c sequences as shown in Tables II may be used as an immunogenic peptide directly (e.g., to screen bacteriophage antibody display libraries or to immunize a rabbit), or may be conjugated to a carrier macromolecule (e.g., BSA) or may compose part of a fusion protein to be used as an immunogen. A preferred NF-AT_c polypeptide comprises the following amino acids sequences:

-NAIFLTVSREHERVGC- (SEQ ID NO: 25);
-PSTSPRASVTEESWLG- (SEQ ID NO: 27);
-SPRVSVTDDSWLGNT- (SEQ ID NO: 33); and
-SHHRAHYETEGSRGAV- (SEQ ID NO: 34);

and may comprise other intervening and/or terminal sequences; generally such polypeptides are less than 1000 amino acids in length, more usually less than about 500 amino acids in length; often spacer peptide sequences or terminal peptide sequences, if present, correspond to naturally occurring polypeptide sequences, generally mammalian polypeptide sequences. One application of the preferred NF-AT_c polypeptide just recited is as a commercial immunogen to raise α-NF-AT_c antibodies in a suitable animal and/or as a commercial immunodiagnostic reagent for quantitative ELISA (e.g., competitive ELISA) or competitive RIA in conjunction with the anti-NF-AT_c antibodies provided by the invention, such as for calibration of standardization of such immunoassays for staging or diagnosis of NF-AT_c-expressing lymphocytic leukemias in humans or cell typing or identification of T cells (such as activated T cells and/or activatable T cells). The preferred NF-AT_c polypeptide just recited will find many other uses in addition to serving as an immunogen or immunological reagent. One or more of the above-listed sequences may be incorporated into a fusion protein with a fusion partner such as human serum albumin, GST, etc. For such fusion proteins in excess of 1000 amino acids, deletions in the fusion partner (albumin) moiety may be made to bring the size to about 1000 amino acids or less, if desired.

In some embodiments, it will be desirable to employ a polyvalent NF-AT_c antigen, comprising at least two NF-AT_c immunogenic epitopes in covalent linkage, usually in peptide linkage. Such polyvalent NF-AT_c antigens typically comprise multiple NF-AT_c antigenic peptides from the same species (e.g., human or mouse), but may comprise a mix of antigenic peptides from NF-AT_c proteins of different species (i.e., an interspecies NF-AT_c polyvalent antigen). Frequently, the spatial order of the antigenic peptide sequences in the primary amino acid sequence of a polyvalent antigen occurs in the same orientation as in the naturally occurring NF-AT_c protein (i.e., a first antigenic peptide sequence that is amino-terminal to a second antigenic peptide sequence in a naturally occurring NF-AT_c protein will be amino-terminal to said second antigenic peptide sequence in a polyvalent antigen. Frequently, spacer peptide sequences will be used to link antigenic peptide sequences in a polyvalent antigen, such spacer peptide sequences may be predetermined, random, or psuedorandom sequences. Spacer peptide sequences may correspond to sequences known to be non-immunogenic to the animal which is to be immunized with the polyvalent antigen, such as a sequence to which the animal has been tolerized. Although many examples of such polyvalent antigens may be given, the following embodiment is provided for illustration and not limitation:

-NAIFLTVSREHERVGC-(aa1) (SEQ ID NO: 25) -AKTDRDLCKPNSLVVEIPPFRN-(aa2) (SEQ ID NO: 31)-GILKLRNSDIELRKGETD- (SEQ ID NO: 37)

where (aa1) and (aa2) are peptide spacers of at least one amino acid and less than 1000 amino acids; aa1 is a peptide sequence selected independently from the aa2 peptide sequence; the length of aa1 (which may be composed of multiple different amino acids) is independent of the length of aa2 (which may be composed of multiple different amino acids).

Immunogenic NF-AT_c peptides may be used to immunize an animal to raise anti-NF-AT_c antibodies and/or as a source of spleen cells for making a hybridoma library from which to select hybridoma clones which secrete a monoclonal antibody which binds to a NF-AT_c protein with an affinity of 1×10^7 M^{-1} or greater, preferably at least 1×10^8 M^{-1} to 1×10^9 M^{-1}. Such immunogenic NF-AT_c peptides can also be used to screen bacteriophage antibody display libraries directly.

One use of such antibodies is to screen cDNA expression libraries, preferably containing cDNA derived from human or murine mRNA from various tissues, for identifying clones containing cDNA inserts which encode structurally-related, immunocrossreactive proteins, that are candidate novel transcription factors or chromatin proteins. Such screening of cDNA expression libraries is well known in the art, and is further described in Young et al., *Proc. Natl. Acad. Sci. U.S.A.* 80:1194-1198 (1983), which is incorporated herein by reference, as well as other published sources. Another use of such antibodies is to identify and/or purify immunocrossreactive proteins that are structurally or evolutionarily related to the native NF-AT_c protein or to the corresponding NF-AT_c fragment (e.g., functional domain; DNA-binding domain) used to generate the antibody. It is believed that such antibodies will find commercial use as such reagents for research applications, just as other antibodies (and biological reagents - such as restriction enzymes and polymerases) are sold commercially.

Various other uses of such antibodies are to diagnose and/or stage leukemias or other immunological disease states, and for therapeutic application (e.g., as cationized antibodies or by targeted liposomal delivery) to treat neoplasia, hyperimmune function, graft rejection, and the like.

An example of an NF-ATc polypeptide is a polypeptide having the sequence:

MPSTSFPVPSKFPLGPAAAVFGRGETLGPAPRAGGTMKSAEEEHYGYASSNVSPALPLPTAHS
TLPAPCHNLQTSTPGIIPPADHPSGYGAALDGCPAGYFLSSGHTRPDGAPALESPRIEITSCL
GLYHNNNQFFHDVEVEDVLPSSKRSPSTATLSLPSLEAYRDPSCLSPASSLSSRSCNSEASSY
ESNYSYPYASPQTSPWQSPCVSPKTTDPEEGFPRGLGACTLLGSPQHSPSTSPRASVTEESWL
GARSSRPASPCNKRKYSLNGRQPPYSPHHSPTPSPHGSPRVSVTDDSWLGNTTQYTSSAIVAA
INALTTDSSLDLGDGVPVKSRKTTLEQPPSVALKVEPVGEDLGSPPPPADFAPEDYSSFQHIR
KGGFCDQYLAVPQHPYQWAKPKPLSPTSYMSPTLPALDWQLPSHSGPYELRIEVQPKSHHRA
HYETEGSRGAVKASAGGHPIVQLHGYLENEPLMLQLFIGTADDRLLRPHAFYQVHRITGKTVST
TSHEAILSNTKVLEIPLLPENSMRAVIDCACILKLRNSDIELRKGETDIGRKNTRVRLVFRVH
VPQPSGRTLSLQVASNPIECSQRSAQELPLVEKQSTDSYPVVGGKKMVLSGHNFLQDSKVIFV

EKAPDGHHVWEMEAKTDRDLCKPNSLVVEIPPFRNQRITSPVHVSFYVCNGKRKRSQYQRFT YLPANGNAIFLTVSREHERVGCFF (SEQ ID NO: 38)

7. Identification and Isolation of Proteins that Bind NF-AT$_c$

Proteins that bind to NF-AT$_c$, NF-ATn, NF-ATc:NF-ATn and/or a NFAT-DNA complex are potentially important transcriptional regulatory proteins. Such proteins may be targets for novel immunomodulatory agents. These proteins are referred to herein as accessory proteins. Accessory proteins may be isolated by various methods known in the art.

One preferred method of isolating accessory proteins is by contacting a NF-AT$_c$ polypeptide to an antibody that binds the NF-AT$_c$ polypeptide, and isolating resultant immune complexes. These immune complexes may contain accessory proteins bound to the NF-AT$_c$ polypeptide. The accessory proteins may be identified and isolated by denaturing the immune complexes with a denaturing agent and, preferably, a reducing agent. The denatured, and preferably reduced, proteins can be electrophoresed on a polyacrylamide gel. Putative accessory proteins can be identified on the polyacrylamide gel by one or more of various well known methods (e.g., Coomassie staining, Western blotting, silver staining, etc.), and isolated by resection of a portion of the polyacrylamide gel containing the relevant identified polypeptide and elution of the polypeptide from the gel portion.

A putative accessory protein may be identified as an accessory protein by demonstration that the protein binds to NF-AT$_c$ and/or a NFAT-DNA complex. Such binding may be shown *in vitro* by various means, including, but not limited to, binding assays employing a putative accessory protein that has been renatured subsequent to isolation by a polyacrylamide gel electrophoresis method. Alternatively, binding assays employing recombinant or chemically synthesized putative accessory protein may be used. For example, a putative accessory protein may be isolated and all or part of its amino acid sequence determined by chemical sequencing, such as Edman degradation. The amino acid sequence information may be used to chemically synthesize the putative accessory protein. The amino acid sequence may also be used to produce a recombinant putative accessory protein by: (1) isolating a cDNA clone encoding the putative accessory protein by screening a cDNA library with degenerate oligonucleotide probes according to the amino acid sequence data, (2) expressing the cDNA in a host cell, and (3) isolating the putative accessory protein. Alternatively, a polynucleotide encoding a NF-AT$_c$ polypeptide may be constructed by oligonucleotide synthesis, placed in an expression vector, and expressed in a host cell.

Yeast two-hybrid systems may be used to screen a mammalian (typically human) cDNA expression library, wherein cDNA is fused to a GAL4 DNA binding domain or activator domain, and a NF-AT$_c$ polypeptide sequence is fused to a GAL4 activator domain or DNA binding domain,

respectively. Such a yeast two-hybrid system can screen for cDNAs encoding proteins which bind to NF-AT_c sequences. For example, a cDNA library can be produced from mRNA from a human mature T cell line or other suitable cell type. Such a cDNA library cloned in a yeast two-hybrid expression system (Chien et al. (1991) *Proc. Natl. Acad. Sci. (U.S.A.)* 88: 9578 or *Cell* 72: 233) can be used to identify cDNAs which encode proteins that interact with NF-AT_c and thereby produce expression of the GAL4-dependent reporter gene. Polypeptides which interact with NF-AT_ccan alos be identified by immunoprecipitation of NF-AT_c with antibody and identification of co-precipitating species. Further, polypeptides that bind NF-AT_c can be identified by screening a peptide library (e.g., a bacteriophage peptide display library, a spatially defined VLSIPS peptide array, and the like) with a NF-AT_c polypeptide. Accessory proteins may also be identified by crosslinking *in vivo* with bifunctional crosslinking reagents (e.g., dimethylsuberimidate, glutaraldehyde, etc.) and subsequent isolation of crosslinked products that include a Lyar polypeptide. For a general discussion of cross-linking, see Kunkel et al. (1981) *Mol. Cell. Biochem.* 34: 3, which is incorporated herein by reference. Preferably, the bifunctional crosslinking reagent will produce crosslinks which may be reversed under specific conditions after isolation of the crosslinked complex so as to facilitate isolation of the accessory protein from the NF-AT_c polypeptide. Isolation of crosslinked complexes that include a NF-AT_c polypeptide is preferably accomplished by binding an antibody that binds a NF-AT_c polypeptide with an affinity of at least $1 \times 10^7\ M^{-1}$ to a population of crosslinked complexes and recovering only those complexes that bind to the antibody with an affinity of at least $1 \times 10^7\ M^{-1}$. Polypeptides that are crosslinked to a NF-AT_c polypeptide are identified as accessory proteins.

Screening assays can be developed for identifying candidate immunomodulatory agents as being agents which inhibit binding of NF-AT_c to an accessory protein (e.g. AP-1) under suitable binding conditions.

8. Methods for Identifying Compounds which Affect NF-AT Activity

These methods of screening may involve labeling NF-ATc, or corresponding peptide with any of a myriad of suitable markers, including radiolabels (e. g., ^{125}I or ^{32}P), various fluorescent labels and enzymes, (e.g., glutathione-S-transferase and β-galactosidase) . If desired for basic binding assays, one of the components may be immobilized by standard techniques, with the non-immobilized component typically being labeled.

The screening assays of the present invention may utilize isolated or purified forms of these assay components. This refers to nucleic acid segments, polypeptides and the like of the present invention which have been separated from their native environment (e.g., a cytoplasmic or nuclear

fraction of a cell, to at least about 10-50% purity. A substantially pure composition includes such compounds that are approaching homogeneity, i.e., about 80-90% pure, preferably 95-99% pure.

While any of the standard pharmaceutical sources of therapeutic candidate agents may be used, a preferred class of agents suitable for use in the screening assays of the present invention are macrolides, particularly those exhibiting a twisted amide peptidyl prolyl bond. See, Schreiber, *Science*, 251, 283-287 (1991) and Banerji et al., *Mol. and Cell. Biol.*, 11, 4074-4087 (1991). These compounds are also preferably capable of binding to and. blocking the cystolic receptors FKBP-12 and FKBP-13. See, Jin et al., *Proc. Natl. Acad. Sci., U.S.A.*, 88, 6671-6681 (1991).

Agent screening using the methods of the present invention can be followed by biological testing to determine if the compound has the desired activities *in vitro* and *in vivo*. The ultimate therapeutic agent may be administered directly to the host to be treated. Therapeutic formulations may be administered in any conventional dosage formulation. While for the active ingredient may be administered alone, preferably, it is presented as a pharmaceutical formulation. Formulations comprise at least one active ingredient as defined above together with one or more pharmaceutically acceptable carriers thereof. Each carrier must be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the patient. Formulations include those suitable for oral, rectal, nasal, or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration of a therapeutically effective dose. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy.

$NF\text{-}AT_c$, $NF\text{-}AT_n$ or fragments thereof produced by proteolytic cleavage or recombinantly, can be used as reagents in heterodimerization assays for identifying agents that disrupt NF-AT complex formation, said agents are thereby identified as candidate immunosuppressant drugs. Alternatively, these polypeptides can be used in *in vitro* assays measuring binding of heterodimeric NF-AT to NF-AT recognition sequences and/or with *in vitro* transcription assays which measure the ability of NF-AT to enhance the rate of transcription of a sequence linked to at least a minimal promoter and an NF-AT recognition sequence. Typically, *in vitro* assays that measure binding of NF-AT to DNA employ double-stranded DNA that contains an array of one or more NF-AT recognition sites. The DNA is typically linked to a solid substrate by any of various means known to those of skill in the art; such linkage may be noncovalent (e.g., binding to a highly charged surface such as Nylon 66) or may be by covalent bonding (e.g., typically by chemical linkage involving a nitrogen position in a nucleotide base, such as diazotization) $NF\text{-}AT_c$ and/or $NF\text{-}AT_n$ are typically labeled by incorporation of a radiolabeled amino acid. The labeled NF-AT protein is contacted with the immobilized DNA under aqueous conditions that permit specific binding in control binding reactions of $1 \times 10^6\ M^{-1}$ or greater (e.g. , 20-150 mM NaCl and 5-100 mM Tris

HCl pH 6-8). Specificity of binding is typically established by adding unlabeled competitor at various concentrations selected at the discretion of the practitioner. Examples of unlabeled protein competitors include, but are not limited to, the following: unlabeled NF-AT_c polypeptide, unlabeled NF-AT_n, polypeptide, bovine serum albumin, and nuclear protein extracts. Binding reactions wherein one or more agents are added are performed in parallel with a control binding reaction that does not include an agent. Agents which inhibit the specific binding of NF-AT protein to DNA, as compared to a control reaction, are identified as candidate immunosuppressants. Also, agents which prevent *in vitro* heterodimer formation of NF-AT and/or prevent transcriptional enhancement by NF-AT *in vitro* are thereby identified as candidate immunosuppressant drugs.

The screening assays of the present invention may utilize isolated, purified, or recombinant forms of these assay components. This refers, e.g., to purified polypeptides and the like of the present invention which have been separated from their native environment (e.g., a cytoplasmic or nuclear fraction of a cell), to at least about 10-50% purity. A substantially pure composition includes such agents that are approaching homogeneity, i.e., about 80-90% pure, preferably 95-99% pure.

8.1. Methods Involving DNA Binding Assays

Candidate immunosuppressants can be identified by NF-AT DNA binding assays. Some candidate immunosuppressants have the ability to inhibit the binding of an assembled NF-AT complex, and, in some instances, of individual NF-AT polypeptides to DNA, particularly where the DNA is double-stranded and has at least one NF-AT recognition site sequence (e.g., as shown in Table I). Various means for detecting specific binding between the NF-AT complex or NF-AT subunit polypeptide and target DNA can be used. Agents which inhibit specific binding of NF-AT complex or NF-AT subunit polypeptide to target DNA are identified as candidate immunosuppressants.

Within the interleukin-2 enhancer (Fig. 2), there are two NF-AT sites, a proximal and distal NF-AT site. The sequence of these is shown in detail in Fig. 3. The essential residues judged by methylation interference are indicated by the lower case letters. Elimination of the NF-AT site from the IL-2 enhancer drastically reduces the ability of the enhancer to function. In addition, arrays of the binding site for the NF-AT protein will direct expression of linked sequences (e.g., reporter or toxin genes) to a specific biologic circumstance, notably the activated T lymphocyte wherein transcriptionally active NF-AT complexes are formed.

A distinguishing feature of the NF-AT DNA binding site upstream of the IL-2 gene is its purine-rich binding site 5'-AAGAGGAAAAA-3' (SEQ ID NO:53). DNA sequence comparisons of the promoter/enhancer regions of several genes that respond to T cell activation signals has

identified putative NF-AT protein binding sites. Such a comparison suggests that NF-AT or a related family member may bind within the promoter/enhancer regions of other T cell activation dependent genes. Most of these genes are sensitive to immunosuppressants, such as FK506 and cyclosporin. A list of putative NF-AT binding sites follows in Table III:

TABLE III

Purine Position Core Sequences	Position	Gene
GAAAGGAGGAAAAACTGTTT (SEQ ID NO: 54)	(-289 to -270)	human IL-2
CCAAAGAGGAAAATTTGTTT (SEQ ID NO: 55)	(-293 to -274)	murine IL-2
CAGAAGAGGAAAAATGAAGG (SEQ ID NO: 56)	(-143 to -124)	human IL-2
TCCAGGAGAAAAAATGCCTC (SEQ ID NO: 57)	(-143 to -124)	human IL-4
AAAACTTGIGAAAATACGTA (SEQ ID NO: 58)	(-71 to -52)	human γ-IFN
TAAAGGAGAGAACACCAGCT (SEQ ID NO: 59)	(-270 to -251)	HIV-LTR
GCAGGGTGGGAAAGGCCTTT (SEQ ID NO: 59)	(-241 to -222)	murine GM-CSF

(Abbreviations: IL-2, interleukin 2; IL-4, interleukin 4; HIV-LTR, human immunodeficiency virus long terminal repeat; GM-CSF, granulocyte-macrophage colony stimulating factor.)

Other NF-AT specific nucleic acid binding sites, usually at least about 10-150 nucleotides (which may be part of a much longer sequence) substantially homologous to these sequences, particularly the NF-AT DNA binding site of the IL-2 enhancer. Ordinarily, such sequences confer NF-AT-dependent transcriptional enhancement on linked (i.e., within 1- 75 kb) promoters and are at least about 80% homologous to the NF-AT DNA binding site, preferably in excess of 90% homologous or more, most preferably are identical.

NF-AT binding assays generally take one of two forms: immobilized target DNA can be used to bind labeled NF-AT protein(s), or conversely, immobilized NF-AT protein(s) can be used to bind labeled target DNA. In each case, the labeled macromolecule (protein or DNA) is contacted with the immobolized macromolecule (respectively, DNA or protein) under aqueous conditions that permit specific binding of the NF-AT protein(s) to the target DNA. Particular

aqueous conditions may be selected by the practitioner according to conventional methods, including methods employed in DNA-protein footprinting and/or *in vitro* nuclear run-on transcription (Dunn et al. *J. Biol. Chem.* 263: 10878-10886 (1988), which is incorporated herein by reference). However, preferable embodiments utilize the following buffered aqueous conditions: 20-150 mM NaCl, 5-50 mM Tris HC1, pH 5-8. It is appreciated by those in the art that additions, deletions, modifications (such as pH) and substitutions (such as KC1 substituting for NaC1 or buffer substitution) may be made to these basic conditions. Modifications can be made to the basic binding reaction conditions so long as specific binding of NF-AT protein(s) to target DNA occurs. Conditions that do not permit specific binding in control reactions (no agent included) are not suitable for use in DNA binding assays.

In embodiments where target DNA is immobilized, preferably double-stranded DNA containing at least one NF-AT recognition site sequence is bonded, either covalently or noncovalently, to a substrate. For example, but not for limitation, DNA can be covalently linked to a diazotized substrate, such as diazotized cellulose, particularly diazophenylthioether cellulose and diazobenzyloxymethyl cellulose (Alwine et al. *Proc. Natl. Acad. Sci. (U.S.A.)* 74: 5350 (1977); Reiser et al. *Biochem. Biophys. Res. Commun.* 85: 1104 (1978); Stellwag and Dahlberg, *Nucleic Acids Res.* 8: 299 (1980), which are incorporated herein by reference).

Alternatively, DNA can be covalently linked to a substrate by partial ultraviolet light-induced crosslinking to a Nylon 66 or nitrocellulose substrate (Church and Gilbert, *Proc. Natl. Acad. Sci. (U.S.A.)* 81: 1991 (1984), which is incorporated herein by reference). Also, for example and not for limitation, DNA can be noncovalently bound to a Nylon 66 or other highly charged anionic substrate (Berger and Kimmel, Methods in Enzymology, Volume 152, Guide to Molecular Cloning Techniques (1987), Academic Press, Inc., San Diego, CA). In some embodiments, it is preferable to use a linker or spacer to reduce potential steric hindrance from the substrate. The immobilized DNA is contacted with labeled NF-AT protein(s), such as NF-AT_n, $NFAT_c$, or fragments of NF-AT_n and NF-AT_c, or complexes thereof (including [NF-AT_c:NF-AT_n] heterodimers).

Preferably, at least one NF-AT protein or NF-AT polypeptide species is labeled with a detectable marker. Suitable labeling includes, but is not limited to, radiolabeling by incorporation of a radiolabeled amino acid (e.g., C^{14}-labeled leucine, H^3-labeled glycine, S^{35},-labeled methionine), radiolabeling by post-translational radioiodination with I^{125} or I^{131} (e.g., Bolton-Hunter reaction and chloramine T), labeling by post-translational phosphorylation with P^{32} (e.g., phosphorylase and inorganic radiolabeled phosphate, calcineurin), fluorescent labeling by incorporation of a fluorescent label (e.g., fluorescein or rhodamine), or labeling by other conventional methods known in the art. In embodiments where the target DNA is immobilized by

linkage to a substrate, at least one species of NF-AT polypeptide is labeled with a detectable marker.

In DNA binding assay embodiments where two or more species of NF-AT subunit polypeptide are used concomitantly, for example a NF-AT_n polypeptide and a NF-AT polypeptide, at least one NF-AT subunit polypeptide species is labeled. Additionally, in some embodiments where more than one NF-AT species are employed, it is preferred that different labels are used for each polypeptide, so that binding of individual and/or heterodimeric and/or multimeric NF-AT complexes to target DNA can be distinguished. For example but not limitation, a NF-AT_c polypeptide may be labeled with fluorescein and a NF-AT_n polypeptide may be labeled with a fluorescent marker that fluorescesces with either a different excitation wavelength or emission wavelength, or both. Alternatively, double-label scintillation counting may be used, wherein one NF-AT subunit polypeptide is labeled with one isotope (e.g.,H^3) and a second NF-AT subunit polypeptide is labeled with a different isotope (e.g., C^{14}) that can be distinguished by scintillation counting using discrimination techniques.

Labeled NF-AT subunit polypeptides are contacted with immobilized DNA target under aqueous conditions as described *infra*. The time and temperature of incubation of a binding reaction may be varied, so long as the selected conditions permit specific binding to occur in a control reaction where no agent is present. Preferable embodiments employ a reaction temperature of at least 20 degrees Centigrade, more preferably 35 to 42 degrees Centigrade, and a time of incubation of at least 15 seconds, although longer incubation periods are preferable so that, in some embodiments, a binding equilibrium is attained. Binding kinetics and the thermodynamic stability of bound NF-AT:DNA complexes determine the latitude available for varying the time, temperature, salt, pH, and other reaction conditions. However, for any particular embodiment, desired binding reaction conditions can be calibrated readily by the practitioner using conventional methods in the art, which may include binding analysis using Scatchard analysis, Hill analysis, and other methods (*Proteins, Structures and Molecular Principles*, (1984) Creighton (ed.), W.H. Freeman and Company, New York).

Specific binding of labeled NF-AT protein to immobilized DNA is determined by including unlabeled competitor protein(s) (e.g., albumin) and/or unlabeled competitor DNA or competitor oligonucleotides. After a binding reaction is completed, labeled NF-AT protein(s) that is specifically bound to immobilized target DNA is detected. For example and not for limitation, after a suitable incubation period for binding, the aqueous phase containing non-immobilized protein and nucleic acid is removed and the substrate containing the target DNA and any labeled protein bound to the DNA is washed with a suitable buffer, optionally containing unlabeled blocking agent(s), and the wash buffer(s) removed. After washing, the amount of detectable label remaining specifically

bound to the immobilized DNA is determined (e.g., by optical, enzymatic, autoradiographic, or other radiochemical methods).

In some embodiments, addition of unlabeled blocking agents that inhibit non-specific binding are included. Examples of such blocking agents include, but are not limited to, the following: calf thymus DNA, salmon sperm DNA, yeast RNA, mixed sequence (random or pseudorandom sequence) oligonucleotides of various lengths , bovine serum albumin, nonionic detergents (NP-40, Tween, Triton X-100, etc.), nonfat dry milk proteins, Denhardt's reagent, polyvinylpyrrolidone, Ficoll, and other blocking agents. Practioners may, in their discretion, select blocking agents at suitable concentrations to be included in DNA binding assays; however, reaction conditions are selected so as to permit specific binding between a NF-AT protein and target DNA in a control binding reaction. Blocking agents are included to inhibit nonspecific binding of labeled NF-AT protein to immobilized DNA and/or to inhibit nonspecific binding of labeled DNA to immobilized NF-AT protein.

In embodiments where protein is immobilized, covalent or noncovalent linkage to a substrate may be used. Covalent linkage chemistries include, but are not limited to, well-characterized methods known in the art (Kadonaga and Tijan, *Proc. Natl. Acad. Sci. (U.S.A.)* 83: 5889-5893 (1986), which is incorporated herein by reference). One example, not for limitation, is covalent linkage to a substrate derivatized with cyanogen bromide (such as CNBr-derivatized Sepharose 4B). It may be desirable to use a spacer to reduce potential steric hindrance from the substrate. Noncovalent bonding of proteins to a substrate include, but are not limited to, bonding of the protein to a charged surface and binding with specific antibodies. DNA is typically labeled by incorporation of a radiolabeled nucleotide (H^3, C^{14}, S^{35}, p^{32}) or a biotinylated nucleotide that can be detected by labeled avidin (e.g., avidin containing a fluorescent marker or enzymatic activity).

NF-AT proteins may exhibit at least three levels of specific binding property: (1) binding to DNA, (2) binding to double-stranded DNA, (3) binding to DNA containing at least one NF-AT recognition site sequence. Each level of binding specificity may be a potential target for candidate immunosuppressants. The DNA assay systems described above may be tailored to assay for each type of binding specificity, if desired.

8.2. Methods for Assaying Heterodimerization

Methods of screening for agents that reduce the binding of the NF-AT_n subunit to the NF-AT_c subunit, and more particularly that prevent the specific heterodimerization of these two subunits, also can identify novel candidate immunosuppressants. Heterodimerization assays involve *in vitro* binding assays comprising NF-AT_n and NF-AT_c polypeptides (native, fragments, or

analogs), wherein test agents can be added to the binding reaction(s) and tested for their ability to inhibit heterodimer formation or reduce the affinity of binding. Agents which interfere with the intermolecular binding between the NF-AT_n subunit (or fragment thereof) and the NF-AT_c subunit (or fragment thereof) are thereby identified as candidate immunosupressants.

These methods of screening may involve labeling NF-AT_n, NF-AT_c, or corresponding fragments or analogs with any of a myriad of suitable markers, including radiolabels (e.g., ^{125}I or ^{32}P) various fluorescent labels and enzymes, (e.g., glutathione-S-transferase, luciferase, and β-galactosidase). If desired for basic binding assays, one of the components may be immobilized by standard techniques. For example but not for limitation, such immobilization may be effected by linkage to a solid support, such as a chromatographic matrix, or by binding to a charged surface, such as a plastic 96-well microtiter dish.

In one class of embodiments, parallel heterodimerization reactions are conducted, wherein one set of reactions serves as control and at least one other set of reactions include various quantities of agents, mixtures of agents, or biological extracts, that are being tested for the capacity to inhibit pairwise heterodimerization between a NF-AT_c polypeptide (native or fragment) and a NF-AT_n polypeptide (native or fragment). Agents that inhibit heterodimerization relative to the control reaction(s) are thereby identified as candidate immunosuppressants.

Preferred embodiments include heterodimerization assays which use NF-AT_n and NF-AT_c polypeptides which are produced by purification from lymphocytes, particularly T lymphocytes (e.g., Jurkat cells).

8.3. Methods Involving *In Vitro* Transcription

Methods of screening for agents that inhibit *in vitro* transcription of template polynucleotides which comprise at least one NF-AT recognition sequence can also be used to identify candidate immunosuppressants. *In vitro* transcription reactions that are dependent on the presence of functional (NF-AT_n:NF-AT_c) heterodimer can serve as the basis for such screening assays. Such screening assays employ purified NF-AT, and NF-AT_n subunits, or fragments thereof, which retain the capacity to form functional [NF-AT_n:NF-AT_c] heterodimers that can interact with NF-AT recognition sequences and enhance *in vitro* transcription of template polynucleotides comprising linked NF-AT recognition sequences and at least a minimal promoter.

NF-AT-dependent *in vitro* transcription reactions are defined herein as reactions wherein the addition of an effective amount of NF-AT heterodimer produces a measurable increase in the amount of transcription product(s) and/or increases the accuracy or frequency of transcriptional initiation as compared to a parallel control reaction which does not contain NF-AT. Thus, NF-AT-dependent transcription is that portion of the total transcription that is attributable to the presence

of NF-AT. Experimental conditions for *in vitro* transcription assays may be selected at the discretion of the practitioner according to methods known in the art, or may be done according to Flanagan and Crabtree, *J. Biol. Chem.* 267: 915 (1992), which is incorporated herein by reference.

Agents which inhibit NF-AT-dependent transcription in such *in vitro* transcription assays are thereby identified as candidate immunosuppressants.

For example and not for limitation, one embodiment of such an *in vitro* transcription assay employs a transcription template that is a polynucleotide comprising at least one NF-AT recognition site linked to a minimal promoter and some additional downstream transcribed sequences. The *in vitro* transcription reaction cocktail comprises the template polynucleotide, an NF-AT_c polypeptide species (native or fragment), an NF-AT_n polypeptide species (native or fragment), an RNA polymerase species, preferably human RNA polymerase II, ribonucleotides, and other constituents which are typically included in transcription reaction cocktails. See Heintz and Roeder, Genetic Engineering (1982) Plenum Press, New York. The reactions may be conducted as described in Flanagan and Crabtree, *J. Biol. Chem.* 267: 915 (1992). Where at least one of the ribonucleotide species is radiolabeled, transcription products of the reaction are electrophoresed on a polyacrylamide gel and autoradiography is performed to identify the size and relative amount(s) of transcription product(s). Parallel *in vitro* transcription reactions are conducted, wherein one set of reactions serves as control and at least one other set of reactions include various quantities of agents, mixtures of agents, or biological extracts that are being tested for the capacity to inhibit (or enhance) *in vitro* transcription of the template. Agents that inhibit the *in vitro* transcription relative to the control reaction(s) are thereby identified as candidate immunosuppressants. Agents which enhance transcription may be novel transcription factors or additional protein factors that participate in NF-AT-mediated transcriptional enhancement.

One preferred embodiment of an *in vitro* transcription assay employs a transcription template comprising the nucleotide sequence which is the 325 nucleotides immediately upstream from the transcriptional start site of the human IL-2 gene.

Another preferred embodiment employs a transcription template that comprises a minimal promoter and a linked tandem array of three NF-AT recognition sequences.

Additionally, preferred embodiments comprise NF-AT_n, and NF-AT_c polypeptides that are produced by purification from lymphocytic cells. Biological activity of NF-AT subunit polypeptides can be modified by altering post-translational modifications, such as phosphorylation. *In vivo*, Ca^{2+} and calmodulin-dependent phosphatase activity, such as calcineurin (Liu et al. *Cell* 66: 807-815 (1991); Friedman and Weissman *Cell* 66: 799-806 (1991), which are incorporated herein by reference), are involved in modulating biological activity of NF-AT. Therefore, in some

embodiments, it is desirable to alter NF-AT polypeptides by post-translational modifications, such as phosphorylation (e.g., with a kinase) or dephosphorylation (e.g., with a phosphorylase).

For example but not for limitation, a NF-AT subunit can be partially phosphorylated by incubation with phosphorylase and inorganic phosphate under conventional reaction conditions known in the art. Alternatively, a NF-AT subunit polypeptide can be partially dephosphorylated by incubation with calf intestinal alkaline phosphatase under conventional reaction conditions. Incubation of NF-AT polypeptides or aggregated NF-AT complex with calcineurin may also be employed. The degree of phosphorylation or dephosphorylation that is achieved with such enzymatic treatments may be determined at the discretion of the practitioner by altering one or more of the following conditions: enzyme concentration, substrate concentration, inorganic phosphate concentration, temperature and duration of incubation, and other reaction parameters known to those of skill in the art.

8.4 Methods for Identifying Compounds which Prevent NF-ATc Nuclear Translocation

Methods for screening compounds that prevent the NF-AT_C component from translocating to the nucleus are preferably based on the observation that immunosuppressants, such as FK506 and CsA, inhibit NF-AT_C from entering the nucleus of FK506 and CsA treated T cells. This inhibition may occur by modifying the NF-AT_C component so that NF-AT_C is unable to engage in entry to the nucleus. Thus, an assay typically involves a polypeptide comprising a peptide region of NF-AT_C which becomes modified (e.g., by phophorylation) upon T cell activation, wherein this polypeptide is used to screen compounds which inhibit or enhance the modification of NF-AT_C all in accordance with standard procedures, such as determining whether or not the modification has occurred by performing polyacrylamide gel electrophoresis on samples obtained subsequent to T cell activation and identifying the relative mobility of NF-AT_C, by immunoreactivity (e.g., Western blotting) and/or autoradiography (e.g., ^{32}P if the modification is phosphorylation).

Alternatively, the nuclear pore of the T cell may be altered to prevent entry of NF-ATc into the nucleus. Such an assay involves analyzing translocation of NF-AT_c, or a corresponding peptide into nuclei that had been previously treated with compounds which alter the nuclear pore of the T cell so that NF-AT_c translocation through or association with the nuclear pore structure fails to occur.

8.5. Methods for Rational Drug Design

NF-AT_c polypeptides, especially those portions which form direct contacts in NF-AT complexes, can be used for rational drug design of candidate NFAT-modulating agents (e.g., antineoplastics and immunomodulators). The substantially purified NF-AT_c and the identification

of NF-AT$_c$ as a docking partner for AP-1 activities as provided herein permits production of substantially pure NF-AT polypeptide complexes and computational models which can be used for protein X-ray crystallography or other structure analysis methods, such as the DOCK program (Kuntz et al. (1982) *J. Mol. Biol.* 161: 269; Kuntz ID (1992) *Science* 257: 1078) and variants thereof. Potential therapeutic drugs may be designed rationally on the basis of structural information thus provided. In one embodiment, such drugs are designed to prevent formation of a NF-AT$_c$ polypeptide: AP-1 polypeptide complex. Thus, the present invention may be used to design drugs, including drugs with a capacity to inhibit binding of NF-AT$_c$ to form NFAT.

Particularly preferred variants are structural mimetics of a dominant negative NF-AT$_c$ mutants, such as a polypeptide consisting essentially of amino acids 1-418 of Fig. 12 and substantially lacking amino acids carboxy-terminal to residue 418. Such mimetics of dominant-negative mutant polypeptides can have substantial activity as antagonists or partial agonists of NF-AT activation (and hence T cell activation).

8.6. Candidate Immunosuppressants

While any of the standard pharmaceutical sources of therapeutic candidate agents may be used, a preferred class of agents suitable for use in the screening assays of the present invention are macrolides, particularly those exhibiting a twisted amide peptidyl prolyl bond. See, Schreiber, *Science*, 251, 283-287 (1991). These agents are also preferably capable of binding to and blocking the cytosolic receptors FKBP-12 and FKBP-13. See, Jin et al., *Proc. Natl. Acad. Sci., U.S.A.*, 88, 6671-6681 (1991).

Agent screening using the methods of the present invention can be followed by biological testing to determine if the agent has the desired activities *in vitro* and *in vivo*. The ultimate therapeutic agent may be administered directly to the host to be treated or administered to explanted cells which may then be returned to the host. Therapeutic formulations may be administered in any conventional dosage formulation. While for the active ingredient may be administered alone, preferably, it is presented as a pharmaceutical formulation. Formulations comprise at least one active ingredient as defined above together with one or more pharmaceutically acceptable carriers thereof. Each carrier must be both pharmaceutically and physiologically acceptable in the sense of being compatible with the other ingredients and not injurious to the patient. Formulations include those suitable for oral, rectal, nasal, or parenteral (including subcutaneous, intramuscular, intravenous and intradermal) administration of a therapeutically effective dose. The formulations may conveniently be presented in unit dosage form and may be prepared by any methods well known in the art of pharmacy.

9. Methods for Forensic Identification

The NF-AT_c polynucleotide sequences of the present invention can be used for forensic identification of individual humans, such as for identification of decedents, determination of paternity, criminal identification, and the like. For example but not limitation, a DNA sample can be obtained from a person or from a cellular sample (e.g. , crime scene evidence such as blood, saliva, semen, and the like) and subjected to RFLP analysis, allele-specific PCR, or PCR cloning and sequencing of the amplification product to determine the structure of the NF-AT_c gene region. On the basis of the NF-AT_c gene structure, the individual from which the sample originated will be identified with respect to his/her NF-AT_c genotype. The NF-AT_c genotype may be used alone or in conjuction with other genetic markers to conclusively identify an individual or to rule out the individual as a possible perpetrator.

In one embodiment, human genomic DNA samples from a population of individuals (typically at least 50 persons from various racial origins) are individually aliquoted into reaction vessels (e.g., a well on a microtitre plate). Each aliquot is digested (incubated) with one or more restriction enzymes (e.g., EcoRI, HindIII, SmaI, BamHI, SalI, NotI, AccI, ApaI, BglII, XbaI, PstI) under suitable reaction conditions (e.g., *see* New England Biolabs 1992 catalog). Corresponding digestion products from each individual are loaded separately on an electrophoretic gel (typically agarose), electrophoresed, blotted to a membrane by Southern blotting, and hybridized with a labeled NF-AT_c probe (e.g., a full-length human NF-AT cDNA sequence of Fig. 12). Restriction fragments (bands) which are polymorphic among members of the population are used as a basis to discriminate NF-AT_c genotypes and thereby classify individuals on the basis of their NF-AT_c genotype.

Similar categorization of NF-AT_c genotypes may be performed by sequencing PCR amplification products from a population of individuals and using sequence polymorphisms to identify alleles (genotypes), and thereby identify or classify individuals.

The following examples are offered by way of example and not by way of limitation. Variations and alternate embodiments will be apparent to those of skill in the art.

Experimental Examples

Example 1: NF-AT is Enriched in Activated T Cells

A DNA binding assay was used to determine the amount of NF-AT present in nuclear extracts from several stimulated and unstimulated cell lines. A radiolabelled oligonucleotide probe

corresponding to the NF-AT binding site was hybridized to concentrated nuclear extracts to determine the amount of NF-AT DNA binding activity present.

Procedure

Nuclear extracts were made according to the procedures of Ohlsson and Edlund (Cell 45:35 (1986)). Briefly, nuclei were extracted with 0.3 M $(NH_4)_2SO_4$ and the fraction that contained the nuclear proteins was precipitated with 0.2 g/ml $(NH_4)_2SO_4$ and dialyzed for 4h. at 4°C. The NF-AT binding site of the IL-2 enhancer (-290 and -263) was used as a probe for binding activity. The binding experiment was carried out essentially as described in Shaw, J.P. *Science* 241:202-205 (1988)).

Results

As shown in Fig. 4, using a simple gel mobility shift assay, a complex forms with the NF-AT DNA-binding sequence and proteins present in nuclear extracts of activated T cells but not with extracts of non-activated T cells or other types of cells. Only the nuclear extract from the Jurkat T cell line that had been stimulated with the T.cell activating agents PMA and PHA contained detectable amounts of NF-AT-specific DNA binding activity.

Example 2: Protein Synthesis is Required for Production of the Nuclear Component of the NF-AT Complex, while the Cytoplasmic Component is Preexisting

To determine whether protein synthesis is required for formation of the nuclear and cytoplasmic components of the NF-AT complex, or whether the proteins are constitutively present in the cells, an NF-AT-specific DNA binding assay was done using NF-AT complex that had been reconstituted from nuclear and cytoplasmic extracts from cells that had been activated in the presence or absence of a protein synthesis inhibitor.

Procedure

NFATZ Jurkat cells were pretreated with 100 μM anisoymycin (Sigma), a protein synthesis inhibitor, for 30 minutes before stimulating the cells. Conditions for activating the cells and preparing nuclear and cytoplasmic extracts are described in Example 3.

Results

As shown in Fig. 5, the protein synthesis inhibitor completely blocked the appearance of the NF-AT complex in activated T cells (Lanes 2,4,6). This demonstrates that the nuclear

component of the NF-AT complex is synthesized de novo upon activation of the T cells. In contrast, cytoplasmic extracts prepared from cells grown in the presence or absence of a protein synthesis inhibitor were able to reconstitute the NF-AT complex (Lanes 3-6). Thus, the cytoplasmic component of the NF-AT complex preexists in the cells prior to stimulation, and additional de novo protein synthesis of NF-AT_C is not required.

Since the activation of the interleukin-2 gene as well as most early T cell activation genes also requires protein synthesis, these observations are consistent with a prominent role for NF-AT in early gene activation.

Example 3: NF-AT Can Be Reconstituted From Cytosolic and Nuclear Subunits

A possible interpretation of the data presented in Fig. 5 is that NF-AT is synthesized but sequestered or compartmentalized within the cell and upon breakage of the cells some transcriptionally active NF-AT is formed. To test this hypothesis, the DNA binding ability of NF-AT complexes reconstituted from cytosolic and nuclear extracts from stimulated and non-stimulated T cells, as well as from cells that had been treated with FK506 just prior to stimulation was tested.

Procedure

NFATZ Jurkat cells and JK12/90.1 cells (a gift from N. Shastri) were stimulated for 2 hours with 20 ng/ml PMA and 2μM ionomycin. To quantitatively block NF-AT formation, FK506 100 ng/ml or CsA (Sandoz) 500 ng/ml were used five minutes prior to the addition of PMA and ionomycin, without any toxic effects to the cells. Nuclear extracts were prepared as described previously with modifications. Cytoplasmic extracts were made from the same cells as the nuclear extracts. Following lysis of the cells with buffer A [10 mM Hepes (pH 7.8), 15 mM Kcl, 2 mM $MgCl_2$, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF] plus 0.05% NP-40, and pelleting of the nuclei, the cytoplasmic fraction was removed and stabilized with 10% (vol/vol) glycerol and 1/10 volume of buffer B [0.3 M Hepes (pH 7.8), 1.4 M Kcl, and 30 mM $MgC1_2$]. The cytoplasmic extract was centrifuged at 200,000 g for 15 minutes. An equal volume of 3 M $(NH_4)_2SO_4$ (pH 7.9) was added to the supernatant, and precipitated proteins were pelleted at 100,000 g for 10 minutes. The pelleted cytoplasmic proteins were resuspended in buffer C [50 mM Hepes (pH 7.8), 50 mM KC1, 1 mM DTT, 0.1 mM EDTA, 0.1 mM PMSF, 10% (vol/vol) glycerol] and desalted by passage over a P6DG column (BioRad). Protein concentrations were determined using a BioRad protein assay kit. To assess the completeness of the nuclear and cytoplasmic fractionation, we assayed for Oct-1 (a constitutive nuclear located DNA binding protein) binding activity and β-galactosidase (cytoplasmic localized) enzyme activity. We found no Oct-1 binding activity in the

cytoplasmic fraction and found that β-galactosidase activity is present in the cytosol at 3.7-fold higher concentration than in the nuclear fraction (data not shown). Electrophoretic mobility shift assays were done essentially as described. Fried, M. and D.M. Crothers NAR 9:6505-6526 (1981). Binding reactions were carried out as previously described. Fiering, S. et al. Genes Dev. 4 1823-1834 (1981). Total amount of protein used in each binding reaction was 10 μg. The end-labelled binding site for NF-AT was derived from the human IL-2 enhancer (-285 to -255 bp). The oligonucleotide sequence is 5'-gatcGGAGGAAAAACTGTTCATACAGAAGGGGT-3' (SEQ ID NO: 61). The mutant NF-AT probe essentially differs from the NF-AT oligonucleotide at four contact guanosine residues. The sequence is 5'-gatcAAGAAAGGAGtAAAAAaTtTTTaATACA GAA-3'(SEQ ID NO: 62). Lower case letters indicate mutated residues. Competition with 10 ng of unlabeled oligonucleotide represents a 100- to 200-fold molar excess over labeled probe.

Results

In the nuclear extracts prepared from stimulated/FK506-treated cells, NF-AT binding activity is reduced substantially and is not observed in the cytoplasmic fractions (Fig. 6A, lanes 3 and 6). Remarkably, binding activity was completely reconstituted by mixing nuclear extracts from stimulated/FK506-treated cells together with cytoplasmic fractions from nonstimulated, or stimulated/FK506-treated cells, neither of which have NF-AT binding activity (Fig. 6A, lanes 7 and 9). Although the mobility of the reconstituted DNA-protein complex is slightly faster than the characteristic mobility of the NF-AT complex, DNA binding specificity is identical. (Fig. 6B, lanes 4-9). Nuclear extracts from nonstimulated cells are not complemented by any of the cytoplasmic extracts (Fig. 6B lanes 1-3) suggesting that stimulation of the cells is essential for synthesis of the nuclear component of NF-AT.

While cytoplasmic extracts from nonstimulated and stimulated/FK506-treated cells can reconstitute the NF-AT complex, cytoplasmic extracts from stimulated cells show only partial reconstitution of NF-AT binding activity (Fig. 6A, lane 8) implying that the cytoplasmic component of NF-AT preexists in nonstimulated cytoplasmic extracts and is translocated to the nucleus following stimulation in the absence of FK506.

We used rapamycin as a control for non-specific effects of FK506. Rapamycin is a structural analog of FK506, and like FK506, contains a structural mimic of a twisted leucyl-prolyl amide bond, binds FK-BP, and inhibits its isomerase activity (Bierer et al. (1990) *Science* 250:556; Bierer et al. (1990) *PNAS (USA)* 87:9231; and Rosen et al. (1990) *Science* 248:863). Despite the fact that rapamycin inhibits isomerase activity, it antagonizes the actions of FK506 on NF-AT-directed transcription, IL-2 gene activation, T cell activation, and programmed cell death (Bierer et al. (1990) *PNAS* 87:9231; Dumont et al. (1985) *J. Immunol.* 134:1599; and Dumont et al.

(1990) *J. Immunol.* 144:1418). Rapamycin did not block translocation of the cytoplasmic component of NF-AT to the nucleus following activation (Fig. 6A, lane 10). This is consistent with its failure to block NF-AT directed transcription (Mattila et al., *supra*).

To determine if impaired nuclear import is also a property of the immunosuppressive prolyl isomerase inhibitor CsA, we teeted the effects of CsA. The biological effects of FK506 and CsA on the immune response are essentially identical (Karrtunen et al. (1991) *PNAS* 88:3872). CsA completely blocks NF-AT directed transcription in T cells and extracts of cells stimulated in the presence of CsA contain less NF-AT binding activity than stimulated controls (Emmel et al. (1989) *Science* 246:1617). Accordingly, mixing nuclear extracts from stimulated CsA-treated cells with cytoplasmic extracts from the sane cells or nonstimulated cells reconstitutes NF-AT binding activity (Fig. 6C, lanes 4-6). Again, nonstimulated nuclear extracts are not able to be complemented by any cytoplasmic extract (Fig. 6C, lanes 1-3). Thus, these results suggest that CsA and FK506 both block the translocation of a pre-existing cytoplasmic component which constitutes part of the NF-AT DNA binding complex.

Example 4: The Cytosolic Form of NF-AT (NF-AT$_c$) is Selectively Expressed in T Cells

Despite the fact that the actions of CsA and FK506 are tissue specific, their binding proteins are ubiquitous (Koletsky et al. (1986) *J. Immunol.* 137: 1056; Kincaid et al. (1987) *Nature* 330:176; Sieklerka et al. (1989) *Nature* 341:755; and Harding et al. (1989) *Nature* 341: 758). This apparent quandary could be rationalized if the drug-isomerase complex acted on a T cell specific molecule. To determine whether the components of the NF-AT complex are found in cell types other than T cells, we tested whether nuclear or cytoplasmic extracts of HeLa cells can be used to reconstitute NF-AT complex alone or in conjunction with extracts from Jurkat cells.

Procedure

HeLa S3 cells were grown in spinner flasks at 37°C in S-MEM (Gibco-BRL) supplemented with 5% fetal calf serum, penicillin (100U/ml), and 100 μg/ml of streptomycin. HeLa S3 were stimulated, nuclear and cytoplasmic extracts were prepared, and gel mobility shift assays were carried out under conditions identical to those described in Figure 6.

Results

HeLa cytoplasmic extracts do not contain NF-AT and homologous mixing of nuclear and cytoplasmic extracts do not contain NF-AT and homologous mixing of nuclear and cytoplasmic extracts from HeLa cells failed to reconstitute NF-AT binding activity (Fig. 7A, lanes 1-9). In contrast, heterologous mixing of Jurkat cytoplasmic extracts with nuclear extracts from HeLa cells

reconstituted NF-AT binding activity (Fig. 7B, lanes 1-3). Furthermore, the reconstituted NF-AT binding activity is specific as demonstrated by oligonucleotide competition (Fig. 7B, lanes 4-9). These results suggest that the oligonucleotide competition (Fig. 7B, lanes 4-9). These results suggest that the nuclear component of NF-AT is present in HeLa cells. In contrast, HeLa cell cytoplasmic extracts cannot reconstitute NF-AT binding activity when mixed with nuclear extracts from stimulated/FK506-treated Jurkat cells (Fig. 7C, lanes 1-3) implying that the cytoplasmic component is T cell specific while the nuclear component of NF-AT is not.

Example 5: Nuclear Import of the Cytosolic Component can be Induced With Ionomycin While Synthesis of the Nuclear Component Requires Only PMA

A unifying feature of the actions of FK506 and CsA is that they inhibit processes which require Ca^{2+} mobilization (Mattila et al. (1990) *EMBO J.* 9:4425; Kay et al. (1989) *Cell. Immun.* 124:175; Cirillo et al. (1990) *J. Immunol.* 144:3891; Gunter et al. (1989) *J. Immunol.* 142:3286). Induction of NF-AT binding and transcriptional activity requires physiologic stimuli that are believed to be mimicked by agents that increase intracellular Ca^{2+} and activate protein kinase C (PKC) (Shaw et al. (1988) *Science* 241:202).

To examine the requirements for induction of the nuclear and cytoplasmic subunits of NF-AT, extracts were prepared from cells stimulated with either PMA alone or ionomycin alone and tested for their ability to reconstitute DNA-binding activity.

Procedure

Gel mobility shifts and preparation of nuclear and cytoplasmic extracts were carried out as described in Example 3.

Results

Cytosolic extracts from ionomycin-treated cells show less ability to reconstitute DNA binding when added to nuclear extracts of stimulated FK506-treated cells than either cytosolic extracts from non-stimulated cells, cytosolic extracts from PMA-stimulated cells or cytosolic extracts from cells stimulated with both PMA and ionomycin (Fig. 8). FK506 treatment did not inhibit PMA/ionomycin-stimulated cells from synthesizing the nuclear component of NF-AT (Fig. 8, Lanes 9-12). Furthermore, mixing cytoplasmic extracts from PMA-stimulated or ionomycin-stimulated cells with nuclear extracts from stimulated/FK506-treated cells fail to reconstitute NF-AT DNA-binding activity (Fig. 8, Lanes 4 and 7), suggesting that the preexisting cytoplasmic

subunit translocated to the nucleus. Thus, CsA and FK506 appear to inhibit the Ca^{2+}-dependent translocation of the cytoplasmic component of NF-AT.

Example 6: FK506 Does Not Inhibit NF-AT-dependent Transcription *In Vitro*

The effect of FK506 on the ability of NF-AT to direct transcription was tested by preparing nuclear extracts from stimulated or stimulated/FK506-treated cells and testing their ability to transcribe a G-less cassette in which transcription was dependent upon three NF-AT sites located within a synthetic promoter.

Procedure

Promoter constructs, nuclear extracts and transcription reactions were prepared as described. NFATZ Jurkat cells, derived from a human T cell leukemia, were stimulated with 20 μg/ml PMA (Sigma), 2 μM ionomycin (Calbiochem) for 2 hours. FK506 was used at 10 ng/ml and added five minutes prior to the addition of PMA and ionomycin. Ribonuclease protection assay of the NF-AT/lacZ mRNA was carried out as previously described. Transcription was quantitated using a radioanalytic imaging system (AMBIS).

Using the human Jurkat T cell line (Wiskocii et al. (1985) *J. Immunol.* 1599), we developed an activation-dependent, T cell specific *in vitro* transcription system which faithfully reflects the complex requirements for IL-2 transcription and more generally T cell activation.

In vitro Transcription Protocol:

Procedure

(i) Cell Culture and Stimulation Conditions

Jurkat cells were grown in RPMI 1640 without L-glutamine, 8% fetal calf serum (FCS) (Irvine Scientific), with penicillin (100 units/ml) and streptomycin sulfate (100 μg/ml) at 5% CO_2 concentrations. Cells were split 1:3 thirty-six hours before stimulation. The morning of the stimulation, the Jurkat cells (1 x 10^6 cells/ml) were centrifuged at 3500 rpm (2000 x g), in a GS-3 rotor for 10 minutes and then resuspended in fresh media to a concentration of 2 x 10^6 cells/ml. In general, 2 μM ionomycin (calbiochem) and 20 ng/ml PMA (Sigma) were used to stimulate the cells. During the 2 hour stimulation, the cells were constantly shaking to prevent the layering of cells on the bottom of the flask.

Hela S3 cells were grown in S-MEM (Gibco) with 8% GCS, with penicillin (100 units/ml) and streptomycin sulfate (100 μg/ml) and 2mM L-glutamine. Hela S3 were stimulated with 20 ng/ml PMA and 2 μM ionomycin.

(ii) Plasmid Construction

The IL-2 G-less plasmid was constructed by fusing the IL-2 enhancer (-326 to +24) to a 377 base pair (bp) G-less cassette generously provided by R. Roeder (Sawadogo and Roeder, 1985) using polymerase chain reaction overlap extension techniques (Horton, R.M. et al. *Gene* 77:61-68 1989; Ho, S.N., et al. *Gene* 77:51-59 1989). The IL-2 enhancer G-less cassette contained on a *Xho I-Bam HI* fragment was inserted into a pUC derivative containing an Xho I site in the polylinker. To avoid PCR artifacts the entire IL-2 enhancer G-less cassette was sequenced. The total size of the IL-2 enhancer G-less transcript is 401 nucleotides (nt). The NF-AT multimer which contains 3 NFAT binding sites (-286 to -257) and NF-IL-2A multimer which contains 4 NF-IL-2A binding sites (-94 to -65) G-less constructs were made by digesting pE3.1 and pA4.1 (Durand et al. 1988) with *Asp 718* and *Bam HI*, respectively, and ligating the fragments into an *Asp 718-Bam El* digested τ-fibrinogen G-less cassette construct. τ-fibrinogen G-less was constructed by fusing -54 to +1 of the τ-fibrinogen promoter (Crabtree, G.R. and Kant, J.A. Cell 31:159-166 1982; Durand et al. 1987) to the 377 bp G-less cassette using PCR overlap extension techniques. All regions of the construct made using PCR technology were sequenced to avoid any point mutations using Sequenase DNA sequencing kit (U.S. Biochemical). The τ-fibrinogen promoter is a minimal promoter containing only a Spl binding and TATA box. Between +1 of the τ-fibrinogen promoter and the G-less cassette a Ssp I restriction enzyme site was inserted. Both the ARRE-2 and ARRE-1 G-less constructs generate 383 nt transcripts.

The HNF-1 (hepatocyte nuclear factor 1) G-less plasmid was constructed by inserting tandemly linked NF-I binding sites from Rat β-fibrinogen promoter (-77 to -65) (Courtois et al. 1987) into Xho-Sal polylinker sites in τ-fibrinogen G-less construct. The adenovirus major late promoter (AdMLP) G-less construct was a generous gift of Drs. M. Sawadoga and R. Roeder. Total size of the AdMLP G-less transcript is 280 nt.

(iii) Preparation of Nuclear Extracts

Jurkat and liver *in vitro* transcription nuclear extracts were essentially made as described by Gorski et al. (Gorski et al. 1986; Maire et al. 1989) with some exceptions. First, the cells were broken in 1.5 M sucrose-glycerol solution to reduce the amount of frictional heat generated during cell lysis. Second 0.5% (vol/vol) nonfat dry milk was added to the homogenization buffer as had been previously described (Maire et al. 1989). Third, the Jurkat nuclei were fractionated on only one 2.0 M sucrose pad preceding salt extraction. Briefly, all manipulations were performed in the cold, and all solutions, tubes, and centrifuges were chilled to 4°C. Protease inhibitors, antipain (1 μg/ml), leupeptin (1 μg/ml), 0.1 mM phenylmethylsulfonyl fluoride (PMSF) and 0.1 mM

benzamidine, were added to all buffers except the dialysis buffer. One mM dithiothreitol was added to all buffers. Following stimulation in the case for Jurkats, the cells (10^9) were centrifuged in a GS-3 rotor, 3500 rpm (2000 x g), for 10 minutes. The media was poured off and the cells were rinsed with 40 mls of phosphate buffered saline. Resuspended pellets were then centrifuged 1000 rpm (200 x g), 10 minutes in a \Beckman GPR tabletop centrifuge. The cell pellet was resuspended in 10 ml of homogenization buffer (10 mM Hepes [pH 7.6] 25 mM KCl, 0.15 mM spermine, 0.5 mM spermidine, 1mM EDTA, 1.25 M sucrose, 10% glycerol (vol/vol), 0.5% nonfat dry milk (vol/vol). An aqueous 0.lg/ml nonfat dry milk solution was centrifuged for 10 minutes in a SS-34 rotor at 1000 rpm (11950 x g) to remove undissolved milk solids before adding to any solution.

The cells were dounced (Teflon-glass homogenizer) until broken using a 1/2 hp drill press (Jet Tools Inc) at high speed. Cells were checked for lysis. Generally, greater than 80% of the cells were lysed. Following lysis, 46 mls of 2M sucrose homogenization buffer (10 mM Hepes (pH 7.6], 25 mM KC1, 0.15 mM spermine, 0.5 mM spermidine, 1mM EDTA, 2M sucrose, 10% glycerol (vol/vol), 0.5% nonfat dry milk (vol/vol) were added to the dounced cells. The homogenized cells (28 mls) were layered on to 10 ml sucrose pads (10 mM Hepes (pH 7.6), 25 mM KC1, 0.15 mM spermine, 0.5 mM spermidine, 1mM EDTA, 2M sucrose, 10% glycerol (vol/vol) and centrifuged at 24,000 rpm for 60 minutes in a SW 28 rotor (103,000 x g).

The pelleted nuclei were resuspended in a total of 6 ml of nuclear lysis buffer (10 mM Hepes [pH 7.6], 100mM KCI, 3mM $MgCl_2$, 0.1 mM EDTA, 10% glycerol (vol/vol).) One ninth volume of 3M $(NH_4)_2SO_4$ pH 7.9 was added and mixed constantly for 30 minutes. The viscous lysate was centrifuge 40,000 rpm, 60 minutes, in a Ti 50 rotor (150,000 x g) to pellet the chromatin.

Following centrifugation, the tubes were quickly removed and the supernatant transferred to another tube before the pelleted chromatin began to reswell. To the supernatant, 0.3 grams of solid $(NH_4)_2SO_4$ per ml were added. The tube was gently mixed for 10 minutes or until all the $(NH_4)_2SO_4$ had gone into solution. The tubes were placed on ice for 40 minutes and gently mixed every 10 minutes. The precipitated proteins were then centrifuged for 15-20 minutes, 40,000 rpm, in a Ti 50 rotor (150,000 x g) . At this point, the pellet was immediately resuspended in dialysis buffer (25 mM Hepes [pH 7.6], 40 mM KC1, 0.1 mM EDTA, 10% glycerol (vol/vol).) Protein extracts from 1 x 10^9 Jurkat cells were resuspended in 200-300 μ1 of dialysis buffer resulting in a final protein concentration of 10 mg/ml. Extracts were dialyzed twice for 2 hours in the cold against 100 volumes of dialysis buffer. During dialysis a precipitate forms that at the end of dialysis was removed by centrifugation in a microfuge (Brinkman Instruments) at a setting of 14 for 5 minutes. Protein concentrations were determined with a Bio-Rad protein assay kit using BSA as

a standard. Protein extracts were frozen in small aliquots on dry ice and immediately stored in liquid nitrogen.

HeLa S3 nuclear extracts were made as previously described (Shapiro, D.J. et al. *DNA* 7:44-45 1988).

(iv) Transcription Reactions

In general, transcription reactions (20 μl) contained 40 μg/ml of circular DNA template [400 ng of the test construct, 40 ng of the AdMLP G-less construct, and 360 ng of poly dI-dC (Pharmacia)] and between 3-5 mg/ml nuclear protein extract in a buffer containing 25 mM Hepes (pH 7.6), 50 mM KC1, 6 mM MgCl2, 0.6 mM each of ATP and CTP, 7 μM UTP, 7 μCi [a-^{32}P] UTP (Amersham, 400 Ci/mmole), 0.5 mM 3'-0-methyl GTP (Pharmacia), 150 units of RNase T1 (BRL), 12 unites of RNase inhibitor (Amersham) and 12% glycerol (vol/vol). EDTA and DTT were contributed by the extract. Transcription reactions using liver or HeLa nuclear extracts contained 40 μg/ml of circular DNA templates (400 ng of the test construct and 400 ng of the AdMLP). All other reaction conditions were kept constant. The reactions were incubated for 45 minutes at 30°C. The transcription reactions were terminated by adding 280 μl of stop buffer (50 mM Tris-HC1 [pH 7.6], 1% SDS, 5 mM EDTA) and were extracted two times with phenol and one time with chloroform. The RNA was precipitated with 15 μg of glycogen, 0.3M sodium acetate (pH 5.2) and 2.5 volumes of ETOH. The pellets were rinsed with 70% ethanol, air dried, and resuspended in 10 μl of loading dye (90% formamide, 0.01% xylene cyanol, and 0.01% bromophenol blue in 1x TBE.) The transcripts were analyzed on 6% denaturing polyacrylamide gels. In general, the gels were exposed overnight at room temperature using XAR-5 (Kodak) x-ray film. Normalized fold induction is calculated by normalizing the amount of transcription from the test G-less construct to that observed from the AdMLP G-less construct and then dividing the amount of test G-less transcription from stimulated nuclear extracts by the amount of test G-less transcription from nonstimulated nuclear extracts. Autoradiograms were quantitated using an Ambis radioanalytic imaging system (Ambis Systems, San Diego, California).

Results

Surprisingly, nuclear extracts from Jurkat cells that had been stimulated for 2 hours with PMA and ionomycin in the presence of FK506 (10 ng/ml) transcribe the IL-2 G-less template at levels nearly equivalent to extracts from fully stimulated Jurkat cells (Fig. 9A, compare lanes 2 and 3) even though transcription of the endogenous IL-2 gene in these cells is fully inhibited (data not shown). Since most of the inhibitory effects of CsA and FK056 on IL-2 gene activation have been shown to be due to the inhibition of NF-AT function (Emmel *supra*; Mattila et al., *supra*), we also

examined transcription directed by this protein. *In vitro* transcription directed by multimerized binding sites for the NF-AT protein was reduced 2.5-fold in nuclear extracts of stimulated/FK506-treated cells (Fig. 9A, compare lanes 5 and 6) despite the fact that NF-AT dependent transcription was totally blocked in the cells used to prepare the extracts (Fig. 9B). In these extracts, NF-AT DNA-binding activity is reduced about 50 to 80% far less than the inhibitory effects on *in vivo* IL-2 gene expression that are generally in excess of 99% (Mattila et al., *supra*), but commensurate with the effects on NF-AT dependent *in vitro* transcription. Thus, it appears that stimulated/FK506-treated cells contain a reduced amount of NF-AT that functions *in vitro* but not *in vivo.*

Example 7: Tandem NF-AT Binding Sites Direct Expression of T Antigen to Activated Lymphocytes in Transgenic Mice

To determine whether a transcriptional promoter under the control of NF-AT regulatory sites will specifically direct expression of a linked gene to activated lymphocytes, we utilized a cell line that contains a construct in which tandem NF-AT binding sites are linked upstream of a gene encoding T antigen (Verweij et al., *J. Biol. Chem.* 265: 15788-15795).

Procedure

Total RNA was isolated from various tissues and cells using guanidium thiocyanate and hot phenol extraction. Equal amounts (10 µg)of RNA were used. RNA mapping experiments with the Sp6/T7 RNA polymerase system were done according to (Melton, D.A. *NAR* 12:7035-7056 (1984)). For mapping correctly initiated NF-AT-Tag mRNA, a SP6 RNA probe was transcribed from *Eco RI* digested pSP6IL-2 vector containing a 117 bp *Xho I-Hind* III fragment (-70 to +47 of NFAT-Tag). Hybridization was allowed to proceed at 42°C for 16 h and samples were digested with 4 ug/ml RNase A and 160 unit s/ml RNase Tl at 30°C for 1 h. Protected fragments were run on a 5% denaturing polyacrylamide gel and exposed to XAR-5 film.

Results

As shown in Fig. 10, only lymphoid cells transcribed the T antigen gene which was under the control of the tandem NF-AT binding sites. Thus, an array of NF-AT binding sites is useful for directing expression of a linked gene specifically to activated lymphoid cells.

Example 8: Activation of NF-AT Probably Requires Phosphorylation

Evidence for phosphorylation of NF-AT was obtained by treating nuclear extracts with calf alkaline phosphatase and examining the mobility of the NF-AT DNA-binding complex on

nondenaturing gels. As shown in Fig. 11, dephosphorylation of NF-AT with calf intestinal phosphatase reduces its ability to associate with its DNA binding site (Fig. 11, lane 4).

Conclusion: Model for the Actions of FK506 and Cyclosporin A: Their Role in Preventing Nuclear Import of NF-AT:

A calcium stimulus induced by the antigen leads to the nuclear import of a subunit of NF-AT. Once in the nucleus, the cytosolic subunit combines with a newly induced nuclear subunit to produce a complex having both DNA-binding activity and transcriptional activity. Neither subunit alone has DNA binding activity and neither subunit alone has transcriptional activity. Cyclosporin A and FK506 prevent the import of the cytosolic component of NF-AT by either preventing the development of competence for nuclear transfer of the cytosolic component of NF-AT or by blocking the appearance of nuclear import signals for NF-AT.

Example 9: Determination of the nucleotide and amino acid sequence of human NF-AT$_c$ cDNA

This example represents the isolation and purification of this novel human NF-AT protein, NF-AT$_c$, the determination of the amino acid sequence of its fragments and the isolation and sequencing of the cDNA clone encoding this protein.

Since our previous work indicated that the cytosolic component of NF-AT was present at relatively low concentrations in human lymphoid cell lines (Northrop et al. (1993) *J. Biol. Chem.* 268: 2917-2923), we chose to purify NF-AT$_c$ from bovine thymus. Accordingly, the protein was purified from bovine thymus glands obtained from newborn calves. Approximately 20 bovine thymuses were homogenized to make a cytosolic extract which was then subjected sequentially to 1) ammonium sulfate precipitation, 2) sulphopropyl Sepharose chromatography, 3) heparin agarose chromatography, 4) affinity chromatography using a multimerized binding site for NF-AT, with the sequence 5'-ACGCCCAAAGAGGAAAATTTGTTTCATACA-3'(SEQ ID NO: 39) coupled to sepharose CL4B, and 5) HPLC on a reverse phase C4 column. The resulting purified protein was subjected to cleavage with LysC/ArgC and fragments isolated by HPLC. The sequences of these individual fragments were then determined by automated Edman degradation. Sequences obtained included: LRNSDIELRKGETDIGR (SEQ ID NO: 35) and LRNADIELR (SEQ ID NO: 40). Degenerate oligos corresponding to GETDIG (SEQ ID NO: 41) (reverse primer) and RNADIE (SEQ ID NO: 42) (forward primer) were made. The degenerate oligo PCR primers had the following sequences:

A forward: (A/C)GIAA(C/T)GCIGA(C/T)AT(A/C/T)GA(A/G) (SEQ ID NO: 43)

A reverse: ICC(A/G/T)AT(A/G)TCIGT(C/T)TCICC (SEQ ID NO: 44)

To isolate the cDNA, oligonucleotide probes were made corresponding to the determined amino acid sequence and used as PCR primers to isolate a 45 base fragment from bovine cDNA prepared from the bovine thymus. The bovine PCR product comprised the nucleotide sequence CTG CGG AAA which encodes -L-R-K-. The same 45 bp fragment can be amplified from human and mouse sources.

This bovine PCR product was then used to screen a cDNA library of the human Jurkat T cell line. Clones were isolated at frequencies of about 1 in 100,000 to 1 in 200,000. A total of five human cDNA clones of various lengths were isolated. Two overlapping clones, one containing the 5' end and one containing the 3' end were ligated together using a unique EcoRI restriction site present in each clone, to produce a full-length cDNA which corresponded in length to the messenger RNA determined by Northern blotting.

The sequence of the NF-AT_c cDNA was determined by the Sanger method and the complete nucleotide and predicted amino acid sequence is shown in Fig. 12. The nucleotide sequence contained 2742 nucleotides and is shown in Figure 12 (SEQ ID NO: 45). The cDNA encodes a protein of 716 amino acids having the amino acid sequence shown in Figure 12 (SEQ ID NO: 38) with a predicted molecular weight of 77,870. An in-frame stop codon upstream from the initiator methionine indicates that the entire NF-AT_c protein is encoded by this cDNA. The initiator methionine indicated in Fig. 12 was determined by fusing this reading frame to a glutathione transferase gene and transfecting the resultant clone into bacteria. The resultant clone produced a fusion protein of the proper molecular weight, indicating that the reading frame designated with the initiator methionine is indeed the correct reading frame. The position of the stop codon was determined by a similar procedure. In addition, the stop codon corresponds to the reading frame for nine of the determined amino acid sequences. A unique repeated sequence of 13 residues was also identified.

The total NF-AT_c protein structure was aligned against individual Rel proteins using a MacIntosh shareware program called DOTALIGN utilizing the alignment parameters of the FASTA programs. Significant homology was observed that corresponded to the Rel domains of these proteins. Enhanced amino acid residue alignment was done using ALIGN from the same suite of programs. Alignment of the Rel similarity regions of NF-AT_c and NF-AT_p was done by hand with no insertions necessary, The Miyata alphabet (Miyata et al. (1979) *J. Mol. Evol.* 12: 214-236) was used to determine similar residues. Fig. 15 shows results of such sequence alignments.

The carboxy-terminal half of NF-AT_c shows limited similarity to the DNA binding and dimerization regions of the Dorsal/Rel family of transcription factors (Fig. 4, for review, Nolan and Baltimore (1992) *Current Biology, Ltd.* 2: 211-220) however, NF-AT_c appears to be the most

distantly related member of the group. There are a significant number of amino acid changes resulting in charge reversals between the Rel family members and NF-AT$_c$, suggesting that charge might be conserved at these positions to maintain salt bridges. Six additional peptides obtained from the purified bovine protein are derived from the bovine homolog of NF-AT$_p$, a cDNA fragment of which was reported by McCaffrey et al. (1993) *Science* 262: 750-754). Comparison of NF-AT$_c$ and NF-AT$_p$ reveals that they are products of distinct genes with 73% amino acid identity in the Rel similarity region (Fig. 15), however, there is very little similarity outside this region. A murine cDNA for NF-AT$_c$ was isolated and the predicted protein was found to be 87% identical to human NF-AT$_c$, and distinctly different from murine NF-AT$_p$.

Example 10: Expression of NF-AT$_c$ in T and non-T cells

The cDNA shown in Figure 12 was fused to the Hemophilus influenza hemaglutinin (HA) 12 amino acid epitope tag in the determined reading frame and operably linked to the SRα promoter in the vector pBJ5 (Lin et al, 1990, *Science* 249:677-679). The resultant construct was transiently transfected by electroporation into Jurkat human T lymphocytes, and into Cos fibroblast cells. Expression of the epitope-tagged NF-AT$_c$ protein was determined by Western blotting of whole cell extracts prepared from the transfected cells, using an antibody (12CA5, Berkeley Antibody Co., CA) that detects the HA epitope.

Fig. 13 shows that NF-AT$_c$ cDNA construct is able to express a protein of appoximately 120 kDA corresponding precisely in size to that of the purified protein, in both Jurkat T cells and Cos cells (see lanes 3 and 6 labeled NF-AT*. Lane 2 shows as control, NF-AT without the epitope tag which cannot be detected in the Western blot).

Example 11: Transfection of NF-ATc Activates Transcription in Both Cos and Jurkat Cells

The NF-ATc cDNA was operably linked to a portion of the SV40 early gene promoter and the HIV transcription regulatory regions in the pBJ vector. This expression vector was co-tranfected into Jurkat and Cos cells with either a) three copies of NF-AT binding site linked to and directing transcription of luciferase (results shown in Fig. 14A and 14B) the entire IL-2 enhancer/promoter directing transcription of luciferase (results shown in Fig. 14B). Cytosolic extracts were prepared and luciferase assays carried out by standard procedures (de Wet et al, 1987, *Mol. Cell. Biol.* 7:724-837).

The results demonstrate that in both Cos cells and Jurkat cells, overexpression of the NF-AT$_c$ protein dramatically enhances NF-AT-dependent transcription by 50-1000 fold (see Fig. 14A).

In addition, overexpression of the NF-ATc protein in Cos cells activates the IL-2 promoter, which in the absence of NF-AT_c cannot otherwise be activated (see Fig. 14B).

These results indicate that the cDNA clone encodes a functional NF-AT_c protein and that NF-AT_c is the protein which restricts expression of interleukin-2 to T cells.

Example 12: NF-AT_c mRNA and Protein Expression

NF-AT_c mRNA is absent in Hela cells (Fig. 16, panel A, lane 7), a cell line incapable of IL-2 or NF-AT-dependent transcription, but is inducible in Jurkat cells (Fig. 16, panel A) . This induction is sensitive to cyclosporin A, (CsA), indicating that NF-AT_c may participate in an auto-stimulatory loop as CsA has been shown to block its nuclear association (Flanagan et al. (1991) *Nature* 352: 803-807). Two B cell lines, muscle tissue, Hep G2 cells and myeloid leukemia cells do not express NF-AT_c mRNA (Fig. 16 panel B). These observations are consistent with the observed T cell-restricted pattern of IL-2 transcription and NF-AT activity. Previous studies (Verweij et al. (1990) *J. Biol. Chem* 265: 15788-15795) revealed NF-AT-dependent transcription predominantly in spleen, thymus and skin of transgenic mice expressing an NF-AT-dependent reporter gene. Consistent with these observations, murine NF-AT_c mRNA shows the same pattern of expression (Fig. 16panel C). Small amounts of NF-AT_c expression are seen in lung and heart, however, this may be due to contamination with circulating T cells. Murine NF-AT_p mRNA, also assayed by quantitative ribonuclease protection, was found to be expressed at approximately equal levels in brain, heart, thymus and spleen (Fig 16 panel C) . In contrast to NF-AT_c, NF-AT_p was not inducible by PMA and ionomycin (Fig 16 panel C).

Methods: Specific human or mouse NF-AT_c or mouse NF-AT_p cDNA fragments were used as templates for the synthesis of RNA transcripts. Ribonuclease protection was done according to Melton et al. (1984) Nucl. Acids. Res. 12: 7035-7056) using 10 μg of total RNA. Splenocytes and thymocytes were isolated and treated as described (Verweij et al. (1990) *J. Biol. Chem* 265: 15788-15795) before isolating RNA, otherwise whole tissue was used.

Example 13: Functional Expression of NF-AT_c

NF-AT luciferase and IL-2 luciferase have been described (Northrop et al. (1993) *J. Biol. Chem.* 268: 2917-2923). β28 luciferase was constructed by inserting a trimerized HNF-I recognition site (β28) in place of the NF-AT recognition sites in NF-AT luciferase. The plasmid pSV2CAT (Gorman et al. (1982) *Mol. Cell. Biol.* 2: 1044-1050) was used as an internal control for transfection efficiency. Cells were transfected with 1.5 μg of luciferase reporter and 3 μg of expression construct as described. After 20 hours of growth, cells were stimulated for 8 hrs. with

20 ng/ml PMA plus or minus 2 μM ionomycin, and harvested for luciferase (de Wet et al. (1987) *Mol. Cell. Biol.* 7: 725-737) and CAT assays (Gorman et al. (1982) *Mol. Cell. Biol.* 2: 1044-1050).

Cos cells were transfected with epitope tagged NF-AT$_c$ as described. Cos cells, Jurkat cells, and murine thymocytes were stimulated for 3hr with PMA and ionomycin. Hela cells were stimulated for 3hr with PMA alone and nuclear extracts prepared as described (Fiefing et al. (1990) *Genes & Dev.* 4: 1823-1834). Cytosols were prepared from non-stimulated Cos cells. Gel mobility shifts were performed as previously described (Flanagan et al. (1991) *Nature* 352: 803-807; Northrop et al. (1993) *J. Biol. Chem.* 268: 2917-2923). Antisera were raised in mice immunized with bacterially expressed glutathione S-transferase fusion proteins using the vector pGEX-3X (Pharmacia) and purified on glutathione agarose. Fusion proteins contained NF-AT$_c$ residues 12 to 143 (immune-1) and 12 to 699 (immune-2).

NF-AT$_c$, expressed in non T cell lines specifically activated transcription from the NF-AT site and the IL-2 promoter, (Fig. 17 panel A (left), and Fig. 17 panel B) . In transiently transfected Jurkat cells, overexpression of NF-AT$_c$ activated an NF-AT-dependent promoter but not an HNF-1 dependent promoter (Fig. 6 panel A (right)) or an AP-1-dependent promoter. Transfection of the NF-AT$_c$ cDNA gives rise to DNA binding activity that is indistinguishable from endogenous NF-AT (Fig. 17 panel C, lanes 1-4). Antibody directed against the HA epitope encoded by the transfected cDNA induces a supershift of the NF-AT complex indicating that NF-AT$_c$ participates in this activity. The nuclear NF-AT activity in transfected Cos cells comigrates with, and has the same binding specificity as, the native nuclear complex in T-cells (Fig. 17 panel C, lanes 4-11). Cytosolic extracts from NF-ATc, transfected Cos cells can reconstitute NF-AT DNA binding activity when mixed with Hela nuclear extract (Fig. 17 panel C, lanes 12-16) as do cytosolic extracts from T-cells (Flanagan et al. (1991) *Nature* 352: 803-807; Northrop et al. (1993) *J. Biol. Chem.* 268: 2917-2923). Antisera raised against bacterially expressed fragments of NF-AT$_c$ that have no similarity to NF-AT$_p$ are able to induce a supershift of the endogenous NF-AT complex, but not the AP-1 complex, from Jurkat cells or thymocytes (immune-1 and immune-2 respectively, Fig. 17 panel D). Immune-2 antisera reduced the DNA-protein complex produced using murine thymic nuclear extracts significantly, consistent with the relatively equal representation of NF-AT$_c$, and NF-AT$_p$ peptides in the purified protein from bovine thymus.

Example 14: NF-AT$_c$ Dominant Negative Mutant Assayed in Transient Transfection Assays

A dominant negative NF-AT$_c$, prepared after extensive deletion analysis of the cDNA, indicated that the amino terminal domain would block NF-AT-dependent function without affecting binding. This region of the cDNA is not found in NF-AT$_p$ and hence can be used to assess the

contribution of NF-AT_c to the activation of the IL-2 gene. The dominant negative NF-AT_c used consists of a carboxy terminal truncation of the epitope tagged NF-AT_c expression plasmid (*supra*) extending to the PvuII site at amino acid 463. Transfection of this dominant negative resulted in more than 90% inhibition of IL-2 promoter function as well as transcription directed by the NF-AT site (Fig 18). This effect was highly specific since transcription directed by the AP-1 site or the RSV promoter and enhancer were relatively unaffected (Fig 18). These results strongly indicate that NF-AT_c contributes substantially to IL-2 gene expression in T cells.

Dominant-negative NF-AT_c polypeptides or peptidomimetics thereof can be used as pharmaceutical antagonists of NF-AT-mediated activation of T cells. In one variation, such drugs can be used as commercial research reagents for laboratory testing and analysis of T cell activation and the like, among many other uses (e.g., immunosuppressant).

Example 15: Post-Translational Modification of NF-AT_c

Post-translational modification of NF-AT_c was investigated in cells treated with agents that activate PKC or increase intracellular Ca++. Cells were transfected with NF-AT_c as described in Fig. 13 and stimulated as shown for 2 hrs plus or minus 100ng/ml CsA. Whole cell lysates were analyzed by western blotting as in Fig. 13. The bulk of NF-AT_c in cells treated with ionomycin migrates faster than that in non-treated cells and this mobility shift is inhibited by CsA (Fig. 19, lanes 1, 3-4). This is consistent with a dephosphorylation event, possibly by direct action of calcineurin (Clipstone and Crabtree (1992) *Nature* 357: 695-697), however, any of a large number of processes could produce the observed mobility changes. There is evidence that NF-AT_p is a substrate for calcineurin, however, the mobility shifts produced by phosphatase treatment of NF-AT_p or NF-AT_c are far greater than those observed in Figure 19. These observations indicate that NF-AT_c is not a direct substrate of calcineurin. PMA treatment produces a slower migrating NF-AT_c (Fig. 19, lane 2); therefore, PKC-activated pathways likely contribute to NF-AT activity by modification of NF-AT_c in addition to activation of the nuclear component.

Equivalents

Although the present invention has been described in some detail by way of illustration for purposes of clarity of understanding, it will be apparent that certain changes and modifications may be practiced within the scope of the claims.